ALKALINE PHOSPHATASE FORMS IN PLASMA: A CLINICAL STUDY

Laila Tibi M.Sc. M.R.C.Path.

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DEDICATION

This thesis is dedicated to my parents.

DECLARATION OF ORIGINALITY

I declare that the work presented in this thesis is my own apart from areas of acknowledged collaboration.

Laila Tibi

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The work for this thesis was carried out part-time, while I was employed as a biochemist by the Lothian Health Board; I am grateful to the Lothian Health Board for allowing me to do this.

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ABSTRACT

The identification of the source of a raised total alkaline phosphatase (ALP; EC 3.1.3.1) activity in plasma, by the measurement of individual ALP forms, is of clinical value although many of the methods available for this purpose are complex, imprecise and non-specific. This thesis has validated, and in some cases modified, available methods for the measurement of the main forms of alkaline phosphatase in plasma: liver, bone, intestinal and high-molecular-mass ALP. The following methods were selected on the basis of their reliability and specificity: polyacrylamide gel electrophoresis, with densitometric scanning, for liver and bone ALP, an enzyme-linked immunosorbent assay (ELISA) for intestinal ALP and ion-exchange chromatography for high-molecular-mass ALP.

These methods were then used to quantify individual ALP forms in specific disease groups and compare activities to those found in healthy adults. The diseases studied (diabetes mellitus and hyperthyroidism) were those where the source of the raised total ALP activity has not been clearly established. Intestinal ALP activity was found to be an important source of the raised total ALP in diabetics of blood group B and O who were secretors. Abnormalities in liver ALP were also present, but these were found mainly in type 2 diabetics. Bone ALP and, to a lesser extent, liver ALP contributed to the raised total ALP activity in patients with hyperthyroidism. These abnormalities were not present in euthyroid patients who were previously hyperthyroid, indicating that they were a temporary feature of the thyrotoxic state.

Specific diseases (chronic renal failure and obstructive liver disease) where the measurement of an individual ALP form was likely to be of more value than total ALP measurement were also studied. In patients with chronic renal failure maintained on haemodialysis, measurements in plasma of activities of total ALP and gamma-glutamyl-transferase identified bone abnormalities in most patients. However, measurement of bone ALP was essential in those patients (16% of the group) who had co-existent liver disease. In patients with obstructive liver disease, the measurement of plasma total ALP activity was of no value in determining the cause of obstruction (intra- or extrahepatic). In these patients, however, a rise in intestinal ALP activity, when measured by ELISA and related to blood group/secretor status category, showed absolute specificity for intrahepatic obstruction.

ABBREVIATIONS

ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
BT	Bromotetramisole
CV	Coefficient of variation
ELISA	Enzyme-linked immunosorbent assay
Eph	Electrophoresis
FPLC	Fast protein liquid chromatography
Free T_4	Free thyroxine
GGT	Gamma-glutamyltransferase
GLA	Gamma-carboxyglutamate
GLU	Glutamate
GST	Glutathione S-transferase
HMM-ALP	High-molecular-mass ALP
25-OH-D	25-hydroxyvitamin D
Le	Lewis
PLAP	Placental alkaline phosphatase
Pptn	Precipitation
PTH	Parathyroid hormone
SD	Standard deviation
SE	Standard error
TSH	Thyroid stimulating hormone
T_3	Tri-iodothyronine

CHAPTER 1

INTRODUCTION

The measurement of total alkaline phosphatase (ALP; EC 3.1.3.1) activity in plasma is one of the most commonly requested biochemical tests performed by routine clinical chemistry laboratories. It is used mainly as an indicator of bone and/or liver disease. Many laboratories also carry out qualitative assessment of ALP forms in plasma to establish the source of the raised plasma total ALP activity. Quantitative measurements of the different ALP forms in plasma are more difficult to perform although they are of clinical value.

This thesis is concerned with the selection and validation of suitable methods for the measurement of the main ALP forms in plasma and the use of these methods, firstly, to define reference ranges for the main ALP forms and, secondly, to quantify these forms in different patient groups. In this introductory chapter, the main forms of ALP will be considered before going on to describe the methods available, at the present time, for their measurement. The clinical value of measurements of individual forms of ALP will be discussed. The specific aims of the thesis will then be outlined.

Alkaline phosphatase catalyses the hydrolysis of phosphate monoesters at an alkaline pH. The precise physiological role of ALP is unknown although it may be involved in the dephosphorylation of phosphotyrosine residues of membrane proteins (Burch et al, 1985). There are four principal isoenzymes of ALP coded by separate genes: the tissue non-specific, intestinal, placental and placental-like isoenzymes (Harris, 1989). As well as the true isoenzymes referred to above, different forms of ALP also exist which arise as a result of post-translational modifications, e.g. the liver, bone and kidney forms of tissue non-specific ALP which differ in their carbohydrate content (Moss, 1986a). The different forms of ALP share many properties in common, in addition to the chemical reactions which they catalyse. They are bound to the cell membrane where they probably exist as tetramers (Hawrylak and Stinson, 1988). "Soluble" forms of ALP also occur e.g. in plasma. These are homodimers, each polypeptide chain having a molecular mass (M_r) of

between 65 and 100 kDa. They contain zinc and are dependent on magnesium for optimal enzymatic activity.

It has been postulated that the four ALP genes have evolved from a single ancestral gene (Harris, 1989) with the first gene duplication giving rise to tissue non-specific ALP and intestinal/placental/placental-like ALP. The gene duplication event, which gave rise to placental ALP from intestinal ALP, is thought to have occurred relatively recently since a placental ALP with properties similar to human placental ALP is found only in higher primates (Goldstein et al, 1982).

The genes encoding three of the proteins (intestinal, placental and placental-like ALP) are linked on the long arm of chromosome 2 while the fourth gene (encoding the tissue non-specific ALP) has been mapped to chromosome 1 (Griffin et al, 1987; Smith et al, 1988). Studies of the four genes at the DNA level have revealed differences between the tissue non-specific ALP gene and the intestinal, placental and placental-like ALP genes. The tissue non-specific ALP gene is at least five times larger than any of the other ALP genes, mainly because of longer introns (Weiss et al, 1988). It also has one more exon (12 in total) compared to 11 exons in each of the other genes.

The placental, placental-like and intestinal ALP show close homology in terms of their amino acid sequences (Table 1.1) while tissue non-specific ALP shows only 52 and 57% positional identity to placental and intestinal ALP respectively (Knoll et al, 1987; Henthorn et al, 1987; Weiss et al, 1986). The enzymes are all synthesised with a signal peptide, which is cleaved off in the mature polypeptide; the signal peptide may be responsible for transporting the newly synthesised enzyme to the membrane. Table 1.1. shows the number of amino acids, derived from cDNA sequences of the coding regions in each case, in the signal peptide and the mature polypeptide for each isoenzyme.

Table 1.1. Amino acid homology of the precursor polypeptide (compared to placental ALP in each case) for placental-like, intestinal and tissue non-specific ALP. The number of amino acids in the signal peptide and mature polypeptide is also given.

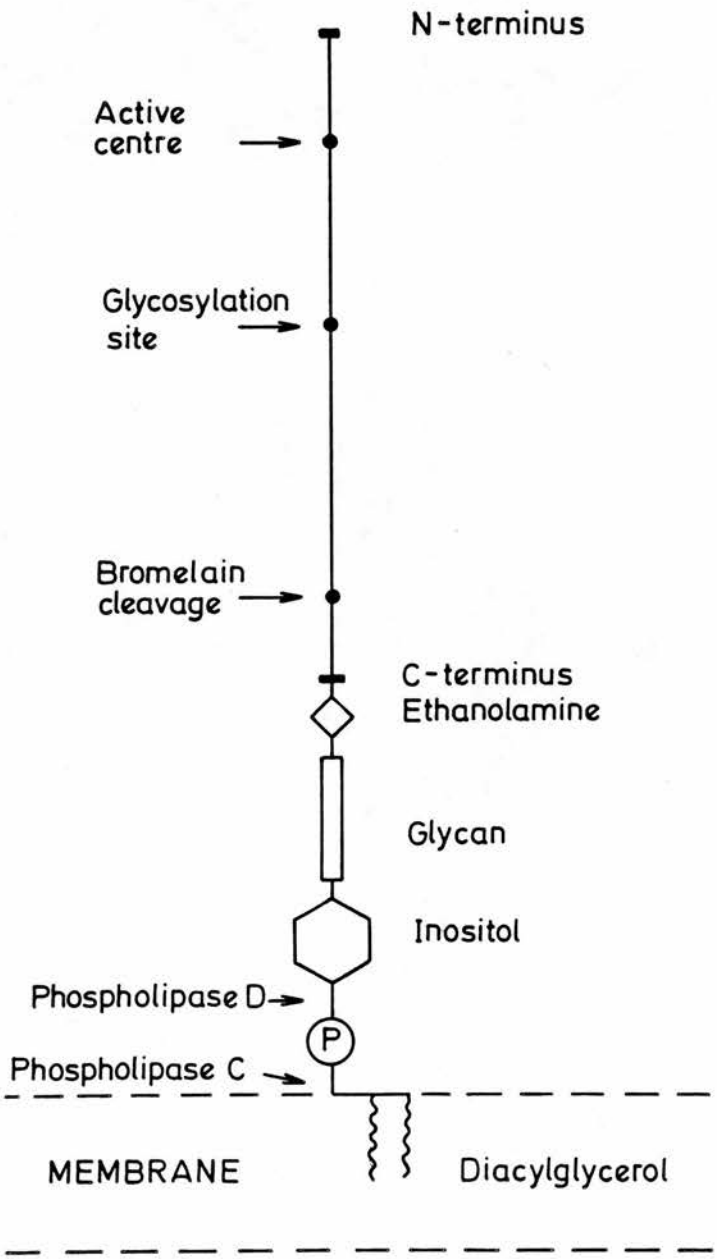
Isoenzyme	Percent homology	Number of amino acids	
		Signal peptide	Mature protein
Placental	100	21	513
Placental-like	98	18	513
Intestinal	90	19	509
Tissue non-specific	52	17	507

All the isoenzymes have a hydrophobic domain at the C-terminal end of the molecule (about 20 - 30 amino acids long). The enzymes are anchored to cell membranes by means of the diacylglycerol portion of phosphatidylinositol; the phosphatidylinositol glycolipid moiety of placental ALP has been characterised (Howard et al, 1987) and is illustrated in Figure 1.1. This is attached to the C-terminal end of the molecule post-translationally.

The phosphatidylinositol glycolipid moiety can be detached from the protein by endogenous membrane-embedded phospholipase C (Malik and Low, 1986) or by phospholipase D circulating in the plasma (Low and Saltiel, 1988). These processes (illustrated in Figure 1.1) yield the "soluble forms" of ALP found in plasma. Other mechanisms for the release of ALP from membranes into plasma include the detergent action of bile salts in the case of liver ALP (Section 1.1.1) and proteolysis in the case of intestinal ALP (Section 1.2).

The patterns of glycosylation, for example the number of sialic acid residues, of the different ALP forms are known to be organ specific. However, little is known about the detailed structure of the carbohydrate moiety although the structure of a single asparagine-linked sugar chain in human placental ALP (at the glycosylation site - Figure 1.1) has recently been reported (Endo et al, 1988).

Figure 1.1. The structure of placental ALP, showing the mature polypeptide chain (see Section 1.3) and the phosphatidylinositol glycolipid region.



1.1. Tissue non-specific alkaline phosphatase

Tissue non-specific ALP is widely expressed and accounts for virtually all of the ALP activity in such tissues as bone, liver or kidney. The liver and bone forms of tissue non-specific ALP are normally present in plasma; raised levels of either or both of these are of diagnostic value. Liver ALP has the same protein backbone as bone ALP but differs in its carbohydrate content which explains its greater electrophoretic mobility and stability to heat and urea compared to bone ALP. Both these forms of ALP have a half-life in plasma of about 2 days and are cleared from the circulation by mononuclear phagocytes of the lymphoreticular system. In cholestatic liver disease, in addition to liver ALP, "particulate" or high-molecular-mass forms (M_r approximately 1000 kDa) are released into plasma; these forms of ALP will be discussed below.

1.1.1. Liver and high-molecular-mass alkaline phosphatase

Alkaline phosphatase activity is concentrated mainly at the exterior canalicular surface of the hepatocyte (Blitzer and Boyer, 1978); from there it is released into bile by the detergent action of bile salts or as a result of membrane recycling and, in the absence of cholestasis, passes into the intestine. Some ALP activity is present at the sinusoidal surface and is released, probably by the action of phospholipase (Kominami et al, 1985), directly into plasma as the relatively hydrophilic low M_r liver ALP (M_r 200 kDa). This low M_r liver ALP is the main component in normal plasma.

In biliary obstruction, there is induction of ALP synthesis by the hepatocyte (Kaplan and Righetti, 1970) with ALP activity becoming more concentrated along the sinusoidal surfaces (Aronsen et al, 1972). Liver ALP, released by phospholipase action, enters directly into plasma where activity of the enzyme is increased. In addition to increased activities of low M_r liver ALP, high-molecular-mass forms also appear in plasma in biliary obstruction. The following mechanisms may be involved in the release of these into plasma:

1. Increased extra- and intracellular bile acid concentrations solubilize the membrane-bound enzyme which is released into plasma where, due to its carboxy-terminal phosphatidylinositol structure, it can form complexes with plasma lipoproteins.

2. Complexes of ALP formed in bile with lipids ("biliary" ALP) can appear in plasma as a result of regurgitation from bile via the "tight" junctions, which become more permeable in cholestasis.
3. Alkaline phosphatase associated with membrane fragments ("koinozymes") may be present in plasma released as a result of hepatocyte damage or destruction (De Broe et al, 1975).

1.1.2. Bone alkaline phosphatase

In bone, ALP is concentrated in the plasma membrane of osteoblasts. These cells are responsible for bone matrix formation. However, the precise role of ALP in the mineralization process is not yet clear: it may be responsible for the local accumulation of phosphate ions needed for bone mineralization, or it may limit the accumulation of inhibitors (inorganic pyrophosphates, nucleotide phosphates) which are known to decrease mineralization.

Bone ALP is released into plasma, probably by the action of a phospholipase (Farley and Jorch, 1983), with its membrane-binding domain (Kalimanovska et al, 1987). Bone ALP is increased in plasma where there is increased osteoblastic activity whether physiological, as in the growing child, or pathological as in Paget's disease. Because the processes of bone resorption and formation are coupled, bone ALP will also be raised in conditions of increased osteoclastic activity (e.g. in osteolytic metastases).

1.1.3. Kidney alkaline phosphatase

In the kidney, ALP is located in the proximal convoluted tubule (Pfleiderer et al, 1984). Alkaline phosphatase of renal origin does not occur in plasma in health but can be present in patients with renal cell carcinoma (Whitaker et al, 1982).

1.1.4. Alkaline phosphatase-immunoglobulin complexes

These are made up of a single immunoglobulin molecule (IgG or occasionally IgA) with two molecules of tissue non-specific ALP (Crofton and Smith, 1978; Maekawa et al, 1985). These complexes are occasionally found in plasma but do not occur consistently in any disease and are of no diagnostic value.

1.2. Intestinal alkaline phosphatase

Intestinal ALP is the predominant isoenzyme in the small intestine, with small amounts of the enzyme present in the kidney (Pfleiderer et al, 1984). Adult intestinal ALP has been shown to have 90% amino acid sequence homology with placental ALP (Henthorn et al, 1987), with which it shares many kinetic and inhibitor properties. It differs from placental ALP and tissue non-specific ALP in that it lacks terminal sialic acid residues.

Intestinal ALP is released from the brush-border of the enterocyte by endogenous proteases which cleave off the membrane-binding domain of the enzyme. It enters the lymphatic system and reaches the general circulation via the thoracic duct. It is rapidly cleared from plasma (half-life of 1 hour) by hepatic galactosyl-glycoprotein receptors (Moss, 1989).

Intestinal ALP appears in plasma after ingestion of fatty meals; the extent of the rise in activity is dependent on the blood group and secretor status of the individual (Langman et al, 1966). Within blood groups B and O, but not group A, individuals who secrete blood group antigens into body fluids, such as saliva (secretors), show higher intestinal ALP activities than non-secretors. Erythrocytes of group A bind intestinal ALP more strongly than erythrocytes of blood groups B and O (Bayer et al, 1980) which may explain why group A secretors do not show higher intestinal ALP activity than non-secretors. Because the rate of clearance of the enzyme is more important than the rate of entry into the circulation, plasma intestinal ALP activities tend to be raised in liver rather than in intestinal disease (Fishman et al, 1965).

Fetal intestinal ALP differs from the adult isoenzyme in its high content of sialic acid. It is present in fetal intestine up to 30 weeks gestation (Mulivor et al, 1978) and can be detected in the serum of premature neonates (Moss et al, 1986). It is probable that a separate gene to that coding for adult intestinal ALP exists for fetal intestinal ALP (Moss, 1989). A variant ALP, the Kasahara isoenzyme (Warnock and Reisman, 1969), which has similar properties to fetal intestinal ALP, has been reported to be present in the plasma of 18% of patients with hepatocellular carcinoma in the United Kingdom (Kay et al, 1982). It is thought that, in a similar way to the Regan (PLAP) and Nagao

(PLAP-like) isoenzymes (Section 1.3), the Kasahara isoenzyme is a result of gene re-expression in cancer, in this case of the fetal-intestinal ALP gene.

1.3. Placental alkaline phosphatase

Placental ALP (PLAP) occurs in the syncytiotrophoblast of term placenta, with traces of PLAP in tissues such as lung and cervix. Placental-like ALP (PLAP-like) is found in low activities in the testis and thymus. Placental ALP is encoded by several allelic genes at a single locus on chromosome 2 (Kam et al, 1985) while the PLAP-like isoenzyme is encoded by a different gene locus (Knoll et al, 1987).

The placental phenotype is determined by the genotype of the fetus and not that of the mother (Robson and Harris, 1967). Thus placental ALP is regarded as a "fetal" isoenzyme. One of the PLAP forms has been sequenced (Millan, 1986); each polypeptide chain is comprised of 513 amino acids (Figure 1.1) with an active site close to the N-terminal end of the chain (residue 92: serine) and one glycosylation site (residue 249: asparagine). It has a hydrophobic domain of 23 amino acids at the C-terminal end, which can be cleaved off by bromelain (Kalimanovska et al, 1987). Both the PLAP and PLAP-like isoenzymes are easily distinguished from other ALP forms by their stability to heat at 65^{o}C.

Placental ALP has a half-life in plasma of 7 days. It is present in plasma in the third trimester of pregnancy and at one time was used as a "placental function test". Certain neoplasms (e.g. ovarian tumours, seminomas) have been found to express PLAP (Regan isoenzyme; Fishman et al, 1968) or PLAP-like isoenzymes (Nagao isoenzyme; Nakayama et al, 1970). The measurement of these isoenzymes using specific immunological techniques can be of value in monitoring treatment of germ cell tumours (Epenetos et al, 1985).

1.4. Analytical methods

Differences in size, charge and patterns of behaviour towards heat and enzyme inhibitors have been used to separate and, in some cases, quantify ALP forms in

plasma. Monoclonal antibodies have been raised to intestinal, placental and tissue non-specific ALP, but antibodies which can distinguish between the liver and bone forms are not yet available.

1.4.1. Electrophoresis

Alkaline phosphatase forms can be separated on cellulose acetate, agarose or acrylamide. Bands of ALP activity are stained with an appropriate substrate which is itself chromogenic or is linked up to a dye and each band quantified by densitometric scanning. However, the separation is not clearly defined for all the ALP forms. Only intestinal ALP, with its slower anodal mobility, is well separated from the other ALP forms and can be quantified by scanning, although immunological methods for its measurement are more sensitive and specific (Section 1.4.5). High-molecular-mass ALP can be measured only on non-sieving media, e.g. cellulose acetate, where it moves ahead of liver ALP; on sieving media, e.g. acrylamide, at the gel concentrations normally used, it will not enter the gel but remains at the origin.

Although the liver and bone forms are poorly separated on most media, the separation, particularly on acrylamide, is adequate for the qualitative assessment of these forms. Separation between these two forms can be improved to allow quantitation in two ways. Firstly, partial treatment of the plasma sample with neuraminidase, prior to electrophoresis, retards the bone form more than the liver form (Moss and Edwards, 1984). The second way involves the addition to the buffer system of wheat germ lectins, which bind to and retard bone ALP (Rosalki and Foo, 1984).

Iso-electric focusing separates plasma ALP forms into numerous bands (Rosendhal et al, 1987) which are difficult to interpret; the technique is of value as a research tool with which the charge properties of the different ALP forms in plasma can be studied.

1.4.2. Heat stability

When plasma is subjected to heat at 65^{0}C for 10 minutes, the remaining activity will be a measure of placental ALP. However, because of the low activities of PLAP and PLAP-like forms in cancer, they are usually measured by more sensitive, immunological techniques (Section 1.4.5). Liver ALP is more stable to heat at 56^{0}C than bone ALP (half-life of bone and liver ALP about 2 and 8 minutes respectively). This difference in heat stability has been used to measure liver and bone ALP activities in plasma (Moss

and Whitby, 1975). However, other ALP forms (intestinal, PLAP and PLAP-like), when present, will interfere. This means that electrophoresis needs to be carried out prior to quantitation by heat-inactivation in order to identify the presence of these other forms. In addition, careful timing and temperature control are needed to obtain consistent results.

1.4.3. Selective inhibition

Inhibitors which distinguish between the intestinal (or placental) isoenzyme and tissue non-specific ALP include L-phenylalanine, which inhibits intestinal ALP, and bromotetramisole, which inhibits tissue non-specific ALP. Both of these inhibitors have been used to measure intestinal ALP in serum (Stolbach et al, 1967; Van Belle et al, 1977). Compared to L-phenylalanine, bromotetramisole gives better discrimination between intestinal and liver/bone forms (Table 1.2).

Urea (Gerhardt et al, 1974) and guanidine hydrochloride (Shephard and Peake, 1986) have been used to differentiate liver and bone forms of ALP. Because these inhibitors are not specific to either the liver or bone form (Table 1.2) and because intestinal or placental ALP may also be present in the same sample, algorithms for the calculation of each ALP form have to be used (Shephard et al, 1986). Accurate pipetting of the inhibitor solution is essential since slight variations in concentration of the inhibitor will over- or under-estimate the activity of each form. The error in estimating ALP forms by selective inhibition methods was recently shown to be high (Tillyer, 1988).

Table 1.2. Residual enzyme activities in the presence of different inhibitors.

Inhibitor	Concentration	Residual enzyme activity (%)		
	mol/L	Intestinal	Liver	Bone
Phenylalanine	0.01	33	86	86
Bromotetramisole	5×10^{-5}	90	10	10
Urea	1.3	-	50	15
Guanidine	0.3	90	47	14

1.4.4. Chromatographic methods

High-molecular-mass ALP has a high negative charge and can be separated readily from other ALP forms by anion exchange chromatography (Crofton and Smith, 1979). It is also possible to separate high-molecular-mass ALP by gel filtration where, because of its large size, it appears in the void volume of the column. However, the charge and size properties of the liver, bone and intestinal forms are too similar for adequate separation by chromatographic means although they have been separated by means of "high performance liquid chromatography" (Schoenau et al, 1986). The use of Reactive Yellow 13 (RY 13) will be mentioned here, although it is not strictly a "chromatographic" separation since it is based on differences in ligand-binding properties of the enzymes. The dye (RY 13), when immobilized on sepharose, will specifically bind intestinal ALP. The enzyme can then be eluted and measured (Williams et al, 1982).

1.4.5. Immunological methods

Monoclonal antibodies to intestinal, PLAP and PLAP-like isoenzymes are available; enzyme-linked immunosorbent assays have been used to measure these forms in plasma (Bailyes et al, 1988; Fisken et al, 1989). Most antibodies raised against the liver or bone forms show almost complete cross-reaction with one another, although antibodies with a five-fold "preference" for the liver form (Lawson et al, 1985) or a two-fold "preference" for the bone form (Seabrook et al, 1988) have been used to measure each form in the presence of the other. Many antibodies to liver ALP do not cross-react with the high-molecular-mass form and can thus be used to quantify this form in plasma (Maguire and Adnan, 1989). An antibody which does not react with liver ALP has been raised against high-molecular-mass ALP (Deng and Parsons, 1988) thus providing a more direct way of measuring the high-molecular-mass form in plasma.

1.5. Clinical value

1.5.1. Total alkaline phosphatase

It was in the early part of this century that raised ALP activities in the plasma of patients with bone or liver disease were first described (Kay, 1929; Roberts, 1930). The measurement of total ALP activity has since then remained a valuable indicator of the presence of liver and/or bone disease.

Plasma total ALP (of liver origin) is markedly raised in cholestatic liver disease, whether intrahepatic (e.g. primary biliary cirrhosis) or extrahepatic (e.g. cholangiocarcinoma). In hepatitis, the activity in plasma is normal or moderately raised. Therefore, as far as plasma levels in liver disease are concerned, increases in total ALP and gamma-glutamyltransferase (GGT; E.C. 2.3.2.2) are more marked in cholestasis, while high levels of alanine aminotransferase (ALT; E.C. 2.6.1.2) and glutathione S-transferase (GST; E.C. 2.5.1.18) occur in hepatocellular damage (Section 1.8). Raised plasma ALP activities, with normal bilirubin levels, may be present in patients with localized biliary obstruction (e.g. by a tumour), since this will stimulate ALP production locally without impairing biliary excretion by the rest of the biliary system.

Very high levels of ALP (of bone origin) are found in the plasma of patients with Paget's disease (osteitis deformans). In this condition, osteoblast activity is increased in an attempt to rebuild bone that is being resorbed by the uncontrolled activity of osteoclasts. Bone secondaries, especially when osteoblastic as in metastases from prostatic carcinoma, also result in high ALP activities. Moderate rises are observed in osteomalacia and hyperparathyroidism (primary or secondary) with skeletal involvement (Wolf, 1986).

The measurement of total ALP activity in plasma is technically easy to perform and can be readily automated, e.g. on the SMAC system (Technicon Instruments Corp., Basingstoke, UK). This means that in a busy routine clinical chemistry department, total ALP may be measured on several hundred plasma samples a day. The main disadvantage of total ALP measurement is its poor specificity for any one disease. Most laboratories will increase the specificity of total ALP by including it in the "liver function test" group, e.g. with bilirubin, ALT and GGT. If total ALP and one or more of the other liver function tests are abnormal, the source of the raised total ALP is assumed to be of liver origin. If, on the other hand, only total ALP is raised, the increase is assumed to be of bone origin. Although this approach may be adequate in most cases, problems can arise in specific situations:

1. An isolated increase in total ALP, due to liver ALP, without an associated abnormality in the other liver function tests can sometimes occur.
2. Patients with metastatic disease in both liver and bone will have an additional increase, due to bone ALP, which will be missed.

3. The interpretation of a raised total ALP activity will be complicated by the presence of significant amounts of intestinal or placental ALP in the same sample.

It is also important to note that, because of the lack of specificity of GGT for liver disease (Section 1.8.4), there is an additional risk in interpreting GGT elevations as pointing to a hepatic origin of ALP (Goldberg, 1980). Thus, GGT correctly identified the source of a raised ALP in only 64% of unselected patients with a raised serum total ALP (Slaunwhite et al, 1978). Problems will arise in patients who abuse alcohol or who are on anticonvulsant drugs. In the latter group, an increased GGT, present as a result of enzyme induction, is often accompanied by an increase in the bone form of ALP (Skillen and Pieredes, 1976). It is therefore safer, where possible, to look at ALP forms specifically using either qualitative or quantitative methods.

1.5.2. Specific ALP forms

Most laboratories use electrophoretic separation, followed by visual assessment of the bands, to determine the source of a raised total ALP activity. These methods give reliable results in samples where the total ALP activity is at least 1.5 times the upper reference limit and where one organ source is dominant (e.g. a strong bone band in Paget's disease). They are less reliable in the following situations where quantitation of each ALP form is of value:

1. Where there is a marginal increase in total ALP activity.
2. Where there is an increase in both the liver and bone forms; this can be difficult to establish by visual assessment.
3. Where changes in the activity of the liver or bone forms over time are being studied, e.g. when monitoring the response to treatment of bone metastases.
4. Where several ALP forms are present in the one sample, e.g. liver, bone, intestinal and high-molecular mass forms. Each form has to be measured specifically to determine which one (or more than one) is responsible for the raised total ALP activity.

The methods available for the measurement of ALP forms (Section 1.4) are complex, time-consuming and, in some cases, unreliable. In general, these methods are not suited for measurements in large numbers of samples. It is probably for this reason

that there is a relatively small number of studies in the literature concerned with the quantitation of ALP forms in health and disease.

1.6. Alkaline phosphatase forms in health

Method-related reference ranges have been reported for liver and bone ALP measured by heat-inactivation (Whitaker et al, 1977), by polyacrylamide gel electrophoresis (Steinberg and Rogers, 1987) and by lectin-affinity electrophoresis (Kuwana et al, 1988). These studies have shown that liver and bone ALP are present in roughly equal activities in the plasma of healthy adults (Whitaker et al, 1977; Steinberg and Rogers, 1987). Kuwana et al, 1988, found that bone ALP, when measured by lectin-affinity electrophoresis, accounted for 70% of total ALP activity in young healthy adults.

Population studies have demonstrated changes in total and bone ALP activities with age during childhood and adolescence (Schiele et al, 1983). The maximum activities for total ALP (up to three times the adult reference range) are found at about 12 years for girls and at 15 years for boys, with adult levels reached at about 17 and 20 years respectively. These variations in total ALP are due almost entirely to changes in bone ALP. During childhood and adolescence, there was a significant correlation between bone ALP and morphometric parameters (height and body weight). Between the ages of 20 and 50 years, there was no substantial variation in plasma total ALP, which however was higher in men than in women for both total and bone ALP. Total ALP activity is increased at the menopause mainly because of a raised bone ALP (Crilly et al, 1980). Liver ALP shows an upward trend with age, males having higher activities than females at all ages (Kuwana et al, 1988). In an elderly population, mean total ALP was raised without any significant clinical abnormality being found (Sharland, 1974). The rise in total ALP reflects an increase in liver rather than bone ALP.

As discussed previously, intestinal ALP activities in plasma depend on both blood group and secretor status of the individual. Secretors of blood groups B and O show a greater rise in plasma intestinal ALP after fat ingestion compared to non-secretors (Kleerekoper et al, 1970). Blood group A secretors behave, in terms of intestinal ALP, in a similar way to non-secretors; both these categories have lower levels than BO secretors. Group AB secretors have intermediate levels of intestinal ALP (Langman et

al, 1966). Therefore, reference ranges for intestinal ALP need to be related to the following blood group/secretor status categories:

1. Secretors of blood groups B and O.
2. Non-secretors plus all blood group A.
3. AB secretors.

The ABO locus has two functional alleles, giving rise to A or B antigens on the red blood cells of group A or B respectively and A and B antigens on group AB. The O gene does not give rise to an antigenic product. In Britain, the majority of the population are groups O and A (47% group O; 42% group A; 8% group B; 3% group AB) (de Gruchy, 1978). Another antigen on the red cells, H substance, is a product of gene H at a locus independent of ABO. H substance is modified by the enzymic products of the A or B genes, resulting in partial or complete elimination of H reactivity, whereas in group O individuals the H specificity on the red cells remains unchanged (Watkins, 1980). The capacity to secrete ABH substances into secretions is determined by a pair of allelic genes (Se and se). Homozygotes (Se Se) or heterozygotes (Se se) are secretors whereas homozygotes for the alternate allele (se se) are non-secretors. Among Europeans about 80% are secretors and 20% are non-secretors. The secretor status of an individual is usually determined on saliva (Section 4.1.3). It is also possible to infer the secretor status from the Lewis (Le) group since the expression of Le a or Le b antigen on the red cell is controlled by the secretor gene. Secretors have the Le b antigen and non-secretors the Le a antigen (Watkins, 1980). About 10% of Europeans are homozygotes for the silent gene (which is the allelic counterpart of the Le gene) and lack both antigens (Le a^-b^-).

From the above discussion, it can be seen that in Britain slightly less than one half of the population (all BO secretors) will have higher intestinal ALP activities than the rest. Most of the studies on intestinal ALP have used visual assessment of the intensity of the intestinal band on electrophoresis and related this to the blood group and secretor status of the individual (Bamford et al, 1965; Langman et al, 1966). In one study, reference ranges were established using a monoclonal antibody capture assay (Bailyes et al, 1988). In this study, however, intestinal ALP activities in 96 blood donors were related only to blood group and not to secretor status (intestinal ALP in blood groups B and O = 0.7 - 14.3 U/L; blood groups A and AB = 0.5 - 7.8 U/L).

High-molecular-mass ALP, when measured by ion-exchange chromatography, accounts for up to 4.3% of total ALP activity in the plasma of healthy adults (Crofton et al, 1979). In another study, where high-molecular-mass ALP was measured by immunoprecipitation, activities of up to 12% of total ALP were found in blood donors (Maguire and Adnan, 1989).

1.7. Alkaline phosphatase forms in disease

This section will review studies in the literature dealing with the measurement of liver, bone, intestinal and high-molecular-mass ALP. Since this thesis is not concerned with the cancer-associated forms (PLAP, PLAP-like and fetal-intestinal isoenzymes), these will not be discussed.

Liver, high-molecular-mass ALP and intestinal ALP have all been used in the biochemical differentiation of the type of liver disease, e.g. malignant or non-malignant, intrahepatic or extrahepatic obstruction. Several studies have considered the value of the measurement of high-molecular-mass ALP in the detection of liver metastases. High-molecular-mass ALP was shown to be sensitive (96%) and specific (93%) for liver metastases and was better than total ALP activity (Viot et al, 1983). Similarly, it was found to be of value in Hodgkin's disease where the presence of the high-molecular-mass fraction was associated with more extensive and progressive forms of the disease (Thyss et al, 1985). A study dealing specifically with the value of high-molecular-mass ALP in the detection of hepatic metastases in lung cancer found a sensitivity of 71% and specificity of 89% (Nishio et al, 1986).

In contrast, other studies have found considerable overlap in high-molecular-mass ALP activities in patients with and without liver metastases. These studies compared activities in patients with liver secondaries to those with other types of liver disease (Crofton et al, 1979; Wenham et al, 1985) or looked at patients with or without secondaries in specific cancers such as breast or colorectal carcinomas (Karmen et al, 1984; Traynor et al, 1986). In the detection of hepatic metastases from breast carcinoma, the test had useful sensitivity (79%) but limited specificity (Karmen et al, 1984). In their study on colorectal cancer, Traynor et al, 1986, conclude that measurement of high-molecular-mass ALP does not offer marked advantages over total

or liver ALP. Discrepancies in the results of these studies may be explained by the different populations selected for study in each case. In practice, however, high-molecular-mass ALP has found no place in the diagnosis or monitoring of patients with liver secondaries (Smith, 1989).

The value of measuring high-molecular-mass and intestinal ALP in plasma for differentiating intra- from extrahepatic causes of obstruction has been assessed by Wenham et al, 1985, and by Warnes et al, 1977. High-molecular-mass ALP was found to be of no value since there was considerable overlap between the two groups, although highest activities were present in extrahepatic obstruction (Wenham et al, 1985). On the other hand, the intestinal band was absent in all cases of extrahepatic disease while, in patients with intrahepatic lesions, an intestinal band was present in 45% of cases (Warnes et al, 1977). A later study found that the specificity of intestinal ALP for intrahepatic obstruction was not absolute even when blood groups of the patients were taken into account. In addition, the test had a diagnostic sensitivity of only 32% (Collins et al, 1987).

Quantitative measurements of liver and bone ALP have been used to identify liver and bone metastases in breast cancer (Mayne et al, 1987). Although both bone and liver ALP had a high specificity for detecting metastases (98% and 97% respectively), they were insensitive. Only 42% of patients with radiologically confirmed bone metastases had a raised bone ALP. Similarly, liver ALP was raised in only 48% of patients with radiological evidence of liver metastases. Serial measurements of bone ALP are useful in monitoring treatment where an increase in activity indicates a favourable response (Moss, 1987).

There is little evidence that quantitative measurements of liver or bone ALP give any more information than total ALP in patients with overt liver disease (e.g. primary biliary cirrhosis) or overt bone disease (e.g. Paget's disease). In fact, most studies have used total ALP, together with radiological investigations, to diagnose and monitor the response to treatment of, for example, bone secondaries from prostatic carcinoma (Bishop et al, 1985). Quantitative studies have been used to establish the source of a raised total ALP in specific diseases. Thus, in thyrotoxicosis, both bone and liver ALP are raised (Rhone et al, 1980). Bone ALP has been found to be raised in other

endocrine disorders such as hyperparathyroidism, acromegaly (Stepan et al, 1978) and diabetes mellitus (Stepan et al, 1980; Maxwell et al, 1986).

High levels of intestinal ALP have been reported in patients with cirrhosis (Fishman et al, 1965), chronic renal failure on haemodialysis (Walker, 1974) and insulin-dependent diabetes mellitus (Skillen et al, 1982). In cirrhosis, the levels are related to blood groups, as they are in healthy people (Stolbach et al, 1967). The presence of raised intestinal ALP activity, however, does not indicate intestinal disease. It has been shown that the incidence of intestinal bands in the plasma of patients with various intestinal-tract diseases is no greater than in normal individuals (Moss, 1989). Intestinal ALP is not elevated in specific intestinal diseases such as active Crohn's disease or ulcerative colitis (Domar et al, 1988).

As mentioned above, intestinal ALP may have a role in the diagnosis of intrahepatic lesions. In other diseases, it is important to recognise the presence of intestinal ALP, whether by qualitative or quantitative methods. This is due to the fact that intestinal ALP, on its own, can be responsible for a raised total ALP which may be wrongly interpreted as evidence of liver or bone disease.

1.8. Liver-related measurements

This section will outline the biochemical measurements used in this thesis, together with liver ALP, to demonstrate the presence of liver abnormalities.

1.8.1. Bilirubin

In hepatobiliary diseases of various causes, bilirubin uptake, storage and excretion may be impaired to varying degrees. Both unconjugated and conjugated bilirubin may be retained in these disorders, giving rise to abnormal plasma levels of total bilirubin. Gross elevations of bilirubin, predominantly conjugated, occur when there is mechanical obstruction of the biliary tree (e.g. carcinoma at the head of the pancreas, choledocholithiasis) while, in conditions where the biliary tree remains patent, levels may be normal or only marginally increased. Raised bilirubin levels can also be present in haemolytic conditions where the increase is in the unconjugated fraction. In general, bilirubin is not as sensitive as enzyme tests in detecting liver disease,

especially if the disease is localized and is therefore not affecting the excretion of bilirubin by the rest of the biliary system.

1.8.2. Alanine aminotransferase

Alanine aminotransferase (ALT; E.C. 2.6.1.2) is a cytosolic enzyme which catalyses the transfer of an amino group between glutamate and pyruvate. ALT is widely distributed (liver, heart, kidney, muscle, etc) but is present at much higher activities in the liver than in other tissues. In liver disease associated with hepatic necrosis (e.g. viral hepatitis), ALT will leak from damaged hepatocytes and can reach levels in the plasma of up to 200 times the upper reference limit.

1.8.3. Glutathione S-transferase

The glutathione S-transferases (GST; E.C. 2.5.1.18) are a group of detoxification enzymes found in many tissues in the body. Four classes of GST have been identified and appear to be encoded by separate families of genes (Mannervik et al, 1985). Three of these are cytosolic and are known as Alpha, Mu and Pi class GSTs; the remaining class is membrane-bound and is called microsomal GST (De Jong et al, 1988). Only the cytosolic forms have been measured in plasma.

The Alpha class GSTs are relatively specific to the liver and kidney. The Alpha class GSTs are dimers composed of 2 sub-units (B_1 and B_2) giving the multiple forms: B_1B_1 (or basic GST), B_1B_2 and B_2B_2 (Stockman et al, 1985). They are released into plasma when hepatocellular damage occurs. Radioimmunoassays which measure the Alpha class GSTs in plasma, particularly those using antisera against the B_1 subunit, have been shown to be of clinical value. In drug-induced liver damage, GST (B_1B_1) is ten times more sensitive in detecting liver damage than the aminotransferases (Beckett et al, 1985a). Similarly, plasma GST measurements in auto-immune chronic active hepatitis were shown to be more sensitive in detecting liver damage than aspartate aminotransferase (AST; E.C. 2.6.1.1) (Hayes et al, 1988).

1.8.4. Gamma-glutamyltransferase

Gamma-glutamyltransferase (GGT; E.C.2.3.2.2) catalyses the transfer of a gamma-glutamyl group from glutathione and other gamma-glutamyl peptides to amino acids, small peptides or water. Histochemical techniques have shown that the enzyme is present in

cell membranes, especially those concerned in secretory or absorptive processes, such as the brush border of the kidney and bile canaliculi. GGT may play a crucial role in the absorption of amino acids from fluids such as the glomerular filtrate and in their uptake into cells from extracellular fluids in general (Goldberg, 1980). This occurs via the gamma-glutamyl cycle which is also responsible for glutathione synthesis (Meister, 1973). In the liver, GGT is located mainly in the smooth endoplasmic reticulum, where it may be involved in drug metabolism.

High concentrations of GGT are found in the kidney and pancreas with a relatively low concentration (but large total quantity) in the liver. Animal experiments have shown that, in the same way as ALP, GGT activity is increased in the liver after bile duct ligation although the peak occurs later than ALP (Kryszewski et al, 1973). In man, highest activities in plasma GGT are found in intra- or extrahepatic obstruction, but there is a large overlap in GGT activities, with the result that GGT has poor discriminatory powers in the differential diagnosis of hepatobiliary disease (Goldberg, 1980). Microsomal GGT is induced by alcohol and drugs such as anticonvulsants. In addition, plasma GGT can be raised in pancreatic, cardiac and renal disease. To sum up, GGT is a sensitive test for detection of liver disease but has poor specificity.

1.9. Bone-related measurements

Normal bone consists of cells (osteocytes) lying in small spaces (lacunae) in an organic matrix and a mineral phase. The organic matrix consists mainly of collagen (90%), the rest being made up of other proteins, peptides, mucopolysaccharides and lipids. The mineral phase consists of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] and several other ions such as sodium, magnesium, carbonate and citrate.

Bone is continually being remodelled by the coupled processes of bone resorption and formation. Two cell types are involved: osteoblasts that derive from connective tissue, and osteoclasts that derive from marrow stem cells (Boyle, 1989). Both cells are found along bone surfaces where remodelling takes place. Osteoblasts eventually become encased in the calcified matrix as osteocytes.

Osteoblasts are thought to initiate bone resorption by secreting proteases such as collagenase. These remove the unmineralized organic material which lines the bone surface. The osteoclast is then able to resorb the naked bone mineral giving rise to a small pit in the bone surface. The resorption phase continues for about 7 to 10 days during which time the osteoclast is controlled by the osteoblast. Receptors for the hormones which increase bone resorption (i.e. parathyroid hormone and 1,25-dihydroxyvitamin D) are found on osteoblasts and not on osteoclasts. Other factors such as interleukin 1, which is one of the most powerful stimulators of bone resorption known (Gowen et al, 1983) may act directly on osteoclasts or via the osteoblasts.

The resorption phase ceases in response to a signal as yet not identified. It is followed by a period in which the lacunae are occupied by mononuclear cells; this phase is known as the reversal phase. The last stage of the sequence is completed by the formation phase which lasts about 3 months. During this period, osteoblast precursors differentiate to give mature osteoblasts; these fill in the resorption pit with osteoid which is then mineralized (Mundy, 1987).

Bone abnormalities can be investigated by radiological means (X-rays and bone imaging), bone histology and biochemical measurements. Measurements of plasma total ALP (or bone ALP specifically) and osteocalcin (Section 1.9.1) have been used to assess osteoblast function and bone formation. Bone resorption has been assessed by measurements of serum tartrate-resistant acid phosphatase (Lau et al, 1987), urinary hydroxyproline (Deacon et al, 1987) or, more recently, urinary pyridinium crosslinks. Pyridinium crosslinks, unlike hydroxyproline, are derived only from bone collagen, and their measurement in urine provides a more specific and sensitive means of assessing osteoclastic activity (Black et al, 1989).

In the clinical studies of this thesis, osteocalcin and parathyroid hormone were used, together with bone ALP, to investigate bone abnormalities. These will be considered in greater detail below.

1.9.1. Osteocalcin

In the mid 1970s, a protein rich in gamma-carboxyglutamate (Gla) residues was identified in calcified tissues and later characterised (Hauschka et al, 1975; Price et al, 1976). This protein was called osteocalcin or bone Gla protein. Osteocalcin is a small

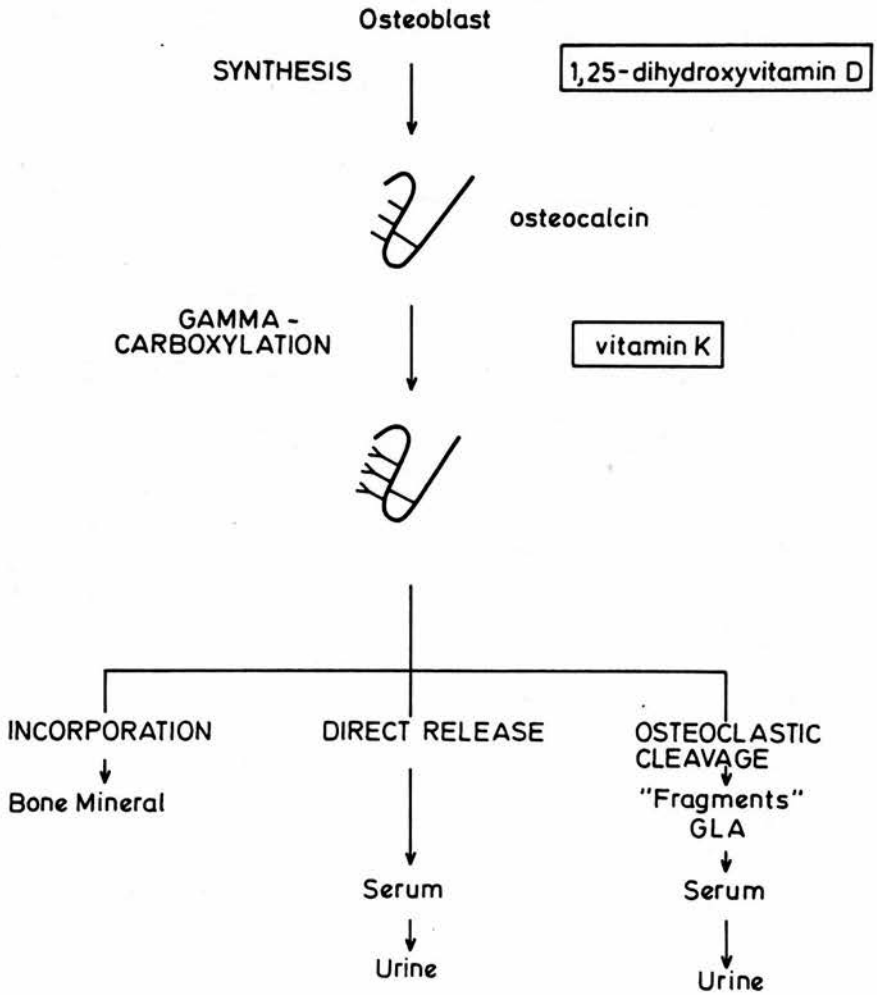
protein (M_r 5,800 daltons) which comprises 1 to 2% of the protein found in bone. Osteocalcin is highly conserved between species. In man, it is made up of 49 amino acids with three Gla residues at positions 17, 21 and 24 (Poser et al, 1980). Osteocalcin also has two cysteine residues which form a disulphide bond between positions 23 and 29.

Specific glutamic acid (Glu) residues on osteocalcin are gamma-carboxylated to form Gla. This occurs post-translationally and involves the vitamin K-dependent enzyme glutamate carboxylase (Figure 1.2). Osteocalcin has a moderate affinity for ionic calcium but binds strongly to hydroxyapatite via its Gla residues. Animals treated with the vitamin K antagonist, warfarin, synthesise non-gamma-carboxylated osteocalcin which does not bind to hydroxyapatite or accumulate significantly in bone (Price and Williamson, 1981).

Osteocalcin is synthesised in the osteoblast where its synthesis is regulated, at the transcriptional level, by 1,25-dihydroxyvitamin D (Figure 1.2)(Pan and Price, 1984). In rat bone, a lowering of osteocalcin levels by the administration of warfarin causes a decrease, in vitro, of bone particle degradation. A smaller number of monocytes attached to osteocalcin-depleted bone, which led to the suggestion that osteocalcin may have a role in attracting bone-resorbing monocytes to bone (Lian et al, 1986).

While osteocalcin is predominantly associated with bone mineral, a small amount is found circulating in blood and can be measured by radioimmunoassay (Price and Nishimoto, 1980). The osteocalcin released into blood represents newly synthesised protein that is not bound to the mineral phase of bone. Osteocalcin is cleared very rapidly from plasma by the kidney with a half-life of about 5 minutes in the rat (Price et al, 1981). Plasma osteocalcin levels are not affected by release of osteocalcin from bone during resorption. This is because bone resorption results in the proteolytic degradation of osteocalcin, giving rise to osteocalcin fragments and gamma-carboxyglutamic acid (Figure 1.2)(Gundberg et al, 1983a) which are normally excreted in the urine (Gundberg and Weinstein, 1986).

Figure 1.2. Synthesis and metabolism of osteocalcin.



A number of radioimmunoassays using polyclonal antibodies against bovine osteocalcin have been developed and some of these are commercially available. These are directed against the C-terminal epitope of bovine osteocalcin, which shows homology with human osteocalcin. Radioimmunoassays based on polyclonal antibodies to human osteocalcin (Taylor et al, 1988) and a monoclonal antibody to bovine osteocalcin (Power et al, 1989) have also been described. The use of different antisera has resulted in a great variation in reported ranges in healthy individuals - up to 32 μg/L in some cases (Power et al, 1989). However, some general points on plasma osteocalcin levels in health can be made. Like bone ALP, osteocalcin levels are raised in children, declining to adult levels at puberty (Gundberg et al, 1983b). Plasma osteocalcin is higher in adult men than in women (Price et al, 1980). In women, osteocalcin falls to a nadir at 35 to 39 years and the mean then rapidly rises two-fold to a plateau from 50 to 75 years. Osteocalcin levels in men fall until the age of 60 to 70 years with little change after that (Worsfold et al, 1988).

In healthy individuals and in patients with bone disease, plasma osteocalcin levels correlate with histological parameters of bone formation (Garcia-Carrasco et al, 1988; Malluche et al, 1984; Brown et al, 1984). Thus, osteocalcin levels are elevated in diseases with increased bone formation and/or turnover. These include hyperparathyroidism (primary or secondary), Paget's disease, cancers with bone metastases (Price et al, 1980) and hyperthyroidism (Garrel et al, 1986). Both normal and raised levels of osteocalcin have been reported in post-menopausal osteoporosis, reflecting the heterogeneity in levels of bone turnover in the populations studied (Brown et al, 1984). Low levels of osteocalcin are found in diseases with reduced bone formation, such as hypoparathyroidism (Price et al, 1980) and hypothyroidism (Martinez et al, 1986), and in patients on glucocorticoid therapy (Lukert et al, 1986). Low levels have also been reported in type 2 diabetes mellitus (Pietschmann et al, 1988) and in chronic liver disease (Diamond et al, 1989); these findings were attributed to reduced bone formation in these diseases.

Most studies have compared plasma osteocalcin with total rather than bone ALP. Significant correlations were found in Paget's disease, hyperparathyroidism and hyperthyroidism (Price et al, 1980; Garrel et al, 1986). When osteocalcin was compared specifically with bone ALP, concordant results were found in hyperparathyroidism, hyperthyroidism, acromegaly and post-menopausal osteoporosis.

Discordant results were found in patients with Paget's disease, osteolytic metastases and chronic renal failure (Duda et al, 1988). In Paget's disease, there may be increased uptake of osteocalcin by abnormal woven bone, resulting in a smaller increase in plasma osteocalcin when compared to bone ALP (Delmas et al, 1986). The reason for lower osteocalcin levels in osteolytic metastases is not clear but a product released by the tumour may inhibit the synthesis of osteocalcin. In renal failure, plasma osteocalcin levels are higher relative to levels found in health than bone ALP activity, this being due to impaired clearance of the smaller-sized osteocalcin molecule. It has been shown that osteocalcin levels rise when the glomerular filtration rate falls below 30 ml/min (Epstein et al, 1985). Moreover, osteocalcin fragments, arising from osteoclastic activity are retained and are measured by some radioimmunoassays (Gundberg and Weinstein, 1986).

1.9.2. Parathyroid hormone

Parathyroid hormone (PTH) is an 84 amino acid peptide secreted by the parathyroid glands. The main stimulus to release of PTH is a reduction in plasma ionized calcium and, conversely, a rise in ionized calcium suppresses the production of PTH. PTH maintains plasma calcium levels within the physiological range by stimulating bone resorption and increasing renal tubular reabsorption of calcium. In addition, PTH increases 1-alpha-hydroxylase activity in the kidney, thus enhancing the conversion of 25-hydroxyvitamin D_3 to 1,25-dihydroxyvitamin D_3. The active metabolite, 1,25-dihydroxyvitamin D_3, increases intestinal calcium absorption.

Parathyroid hormone undergoes proteolytic cleavage, mainly in the liver, to give amino- and carboxy-terminal fragments which are then released into the circulation. Only the intact peptide and the amino terminal fragment are biologically active (Silverman and Yalow, 1973). A variety of immunoassays have been developed, but many of these cannot distinguish the intact peptide from the fragments. Recently, two-site immunoradiometric assays have been described which allow specific measurement of intact PTH (Nussbaum et al, 1987).

In addition to increasing osteoclastic activity and raising plasma calcium levels, PTH also increases osteoblastic activity. This raises bone ALP activity and osteocalcin concentrations in plasma. This pattern of results may be found in primary hyperparathyroidism when there is bone involvement. Raised plasma calcium levels

resulting from conditions unrelated to parathyroid pathology, such as hypercalcaemia of malignancy, will give suppressed or undetectable PTH levels. Sensitive immuno-radiometric assays are able to distinguish between the low serum PTH levels found in these patients and the levels found in healthy individuals (Nussbaum et al, 1987).

Most patients with advanced renal failure have secondary hyperparathyroidism, and some may go on to develop tertiary hyperparathyroidism. In renal failure, the biologically-inactive carboxy-terminal fragments are retained. The immunoradiometric assays, mentioned above, will measure only the biologically-active intact peptide in the serum of these patients. These measurements, therefore, may be of value in the investigation of parathyroid disease and the associated osteodystrophy of patients with renal failure.

1.10. Aims of the thesis

The main aims of this thesis are as follows:

1. To assess and validate available methods for the quantitation of the main ALP forms in plasma (liver, bone, intestinal and high-molecular-mass ALP; Chapter 2). To select methods, on the basis of their reliability and suitability for large sample numbers, for use in subsequent clinical studies.

2. To measure the main ALP forms in the plasma of healthy individuals using the selected methods (Chapter 5). In the case of intestinal ALP, to relate the activity to blood group and secretor status.

3. To measure ALP forms in selected groups of patients (see below) and compare activities in patients with those found in healthy individuals. To assess the diagnostic value of these findings.

4. To correlate liver ALP activity in these groups to other liver-related biochemical measurements (plasma bilirubin, ALT, GST and GGT).

5. To correlate bone ALP activity in these patients to other bone-related biochemical measurements (calcium and osteocalcin in plasma, PTH in serum). Where necessary to develop methods for measurement of other diagnostic variables to be used in the studies on alkaline phosphatase e.g. osteocalcin (Chapter 3).

Individual ALP forms will be measured in the following disease groups:

1. Diseases where the source of raised total ALP in plasma is not clearly established. The diseases studied will be diabetes mellitus and hyperthyroidism. In both cases, there is controversy regarding both the extent of increase in total ALP activity and the source of the raised activity. Clinical and biochemical abnormalities relating to liver and bone can be present in diabetes and in hyperthyroidism. These will be discussed in Chapters 6 and 7.

2. Diseases where specific measurement of one ALP form in plasma may be of more value than the measurement of total ALP. The following ALP forms will be studied:

a. Bone ALP measurement, rather than total ALP, in the investigation of bone disease in patients maintained on haemodialysis (Chapter 8).
b. Intestinal ALP and high-molecular-mass ALP in the differential diagnosis of obstructive liver disease (Chapter 9).

CHAPTER 2

MEASUREMENT OF ALKALINE PHOSPHATASE FORMS

This chapter concerns the assessment and validation of the methods which were used to measure liver, bone, intestinal and high-molecular-mass ALP activities in plasma for the clinical studies which are presented in this thesis.

2.1. MATERIALS

The materials used are listed below subdivided for convenience into sections which correspond to the various measurements made.

Total alkaline phosphatase

Alkaline phosphatase (AMP-Buffer) C-system a for automated and manual analysis (cat. no. 396 494): Boehringer Mannheim, Bell Lane, Lewes, UK.
p-Nitrophenyl phosphate, disodium salt 5 mg/tablet (product no. N9389): Sigma Chemical Co., Fancy Road, Poole, Dorset, UK. Diethanolamine (AnalaR grade): British Drug Houses (BDH), Poole, Dorset, UK.

Liver and bone alkaline phosphatase

Corning electrophoretic method; Tris Bicine Buffer set (cat. no. 470187), Agarose Special Electrophoresis Film (cat. no. 470104), AP Isoenzyme Substrate set (cat. no. 470073): Corning Medical, Halstead, UK.
Polyacrylamide gel electrophoretic method; acrylamide, NN'-methylene-bisacrylamide, ammonium persulphate, NNN'N'-tetramethylethylenediamine (TEMED): BDH.
α-Naphthyl acid phosphate potassium salt, 4-aminodiphenylamine diazonium sulphate: Sigma.
Neuraminidase from vibrio cholerae (product no. 39027): BDH.
Lectin (Triticum vulgaris - wheat germ, product no. L9640): Sigma.

Anion exchange column; Mono Q HR 5/5 (code no. 17-0546-01): Pharmacia, Milton Keynes, UK.

Intestinal alkaline phosphatase

Inhibitors; L-p-bromotetramisole oxalate (product no. B8007), L-phenylalanine (product no. P2126): Sigma.

Antibodies; monoclonal antibodies to intestinal ALP (AAP-1) and to liver/bone ALP (TRA 254/2) from Sir Walter Bodmer, Imperial Cancer Research Fund, Lincoln's Inn Fields, London. Rabbit anti-mouse immunoglobulin (code Z109): Dakopatts, High Wycombe, UK. Sheep anti-mouse immunoglobulin (product code S201-220): Scottish Antibody Production Unit (SAPU), Law Hospital, Carluke, Lanarkshire, UK.

Solid phase; Sephacryl S-500: Pharmacia.

High-molecular-mass alkaline phosphatase

Agarose Type 1 Low EEO (product no. A6103): Sigma.

Ion exchange cellulose (DE 52): Whatman Biosystems Ltd, Maidstone, Kent, UK.

Gel filtration column; Superose 6 (code no. 17-0537-01): Pharmacia.

Placental alkaline phosphatase

Rabbit anti-human placental alkaline phosphatase (code A268): Dakopatts.

Miscellaneous

All other reagents were ordered from BDH or Sigma. All buffers were made up using AnalaR grade reagents from BDH.

2.2. EQUIPMENT

Centrifugal analyser: Cobas Bio (Roche).

Electrophoresis tanks (horizontal: Corning, vertical: LKB).

Scanners: Appraise (Beckman), Corning 720.

Chromatography: FPLC (Pharmacia).

Plate washer (Amersham); plate reader: Titertek Multiscan (Flow Laboratories).

Flame photometer (Instrument Laboratories 943).

2.3. MEASUREMENT OF TOTAL ALKALINE PHOSPHATASE

A method using p-nitrophenyl phosphate in 2-amino-2-methyl-1-propanol (AMP) buffer was used to measure total ALP activities in plasma (Bowers and McComb, 1975). Absorbance change at 405 nm was monitored at 37°C on a Cobas Bio centrifugal analyser. The final conditions in the cuvette were as follows: AMP buffer, 0.9 mol/L, pH 10.5; p-nitrophenyl phosphate, 16 mmol/L; Mg^{2+}, 1.0 mmol/L. A molar absorption coefficient of 1860 $m^2 mol^{-1}$ was used for the calculation of activities. A list of parameter settings for the Cobas Bio for total ALP activity measurement is given in Table 2.1.

Table 2.1. Parameter listing for total ALP activity measurement on the Cobas Bio.

Units	U/L
Calculation Factor	1350
Limit	0.55
Temperature (°C)	37
Wavelength (nm)	405
Sample volume (μl)	10
Diluent volume (μl)	20
Reagent volume (μl)	230
Time of first reading (sec)	90
Time interval (sec)	10
Number of readings	12

When a sensitive method was required for ALP activity measurements (e.g. for fractions collected from columns) 1 mol/L diethanolamine buffer was used instead of AMP buffer. All measurements of total ALP were carried out in duplicate. The between-batch precision for total ALP measurement was 3.4% (mean = 73 U/L, SD = 2.5, range 23-132 U/L).

2.4. MEASUREMENT OF LIVER AND BONE ALKALINE PHOSPHATASE

Liver and bone ALP will be considered together in this section since all the methods used measured both forms at the same time. The following methods were tried for the quantitation of liver and bone ALP:

1. Heat-inactivation
2. Agarose electrophoresis
3. Chromatography: FPLC
4. Lectin-precipitation
5. Polyacrylamide gel electrophoresis

A polyacrylamide gel electrophoretic method was finally chosen as the most suitable method in terms of precision, accuracy and suitability for large numbers of measurements. In this section, a brief account will be given of methods 1 - 4, with a fuller description of the assessment and validation of the polyacrylamide gel method.

2.4.1. Heat-inactivation

Introduction

The heat-inactivation method (Moss and Whitby, 1975) is based on differences in stability of plasma liver and bone ALP to heat at 56°C. Alkaline phosphatase activities were measured at two time intervals (15 and 25 minutes). The logarithm of percentage residual activity (y axis) was plotted against time (x axis) and an estimate of liver ALP was obtained by extrapolating to zero on the y axis. The method assumes that ALP forms other than liver and bone ALP are not present in the plasma sample. Bone ALP was therefore calculated from (total - liver ALP).

Method

Plastic Cobas cups were used for heat-inactivation experiments. Distilled water or plasma in these cups reached the water bath temperature of 56°C in 1 minute. 360 µl of plasma were added, in duplicate, to pre-warmed Cobas cups in a water bath at 56°C. At precisely 15 and 25 minutes, 150 µl of plasma were removed from each cup in the water bath and added to cups pre-cooled in ice. The temperature of the

water bath was monitored throughout the inactivation period using a thermistor and was 56 ±0.3°C. Alkaline phosphatase activities at 15 and 25 minute time points were measured using the same conditions as for total ALP activity but increasing the sample volume to 20 µl for the 15 minute sample and 60 µl for the 25 minute sample to give more precise measurements of the low activities remaining.

Liver ALP activity was calculated from plots of logarithm of the residual activity against time; bone ALP activity was obtained by difference. The between-batch precision for the heat-inactivation method was checked by repeat measurements of liver and bone ALP activities in different plasma samples in 15 separate batches. To check for interference by other ALP forms, liver and bone ALP were measured in the following samples:

> Sample 1 had a strong intestinal band [quantitative results for ALP forms (Section 2.4.5, Section 2.5.3 and Section 2.6.2): liver ALP = 58 U/L, bone ALP = 114 U/L, intestinal ALP = 158 U/L, high-molecular-mass ALP = 10 U/L]. Sample 2 had mainly liver and high-molecular-mass ALP present (quantitative results for ALP forms: liver ALP = 904 U/L, bone ALP = 0 U/L, intestinal ALP = 4 U/L, high-molecular-mass ALP = 125 U/L).

Results

Table 2.2 gives the between-batch precision for liver ALP and for bone ALP measurements. Sample 1 gave a liver ALP activity of 116 U/L and a bone ALP activity of 224 U/L (total ALP activity = 340 U/L). Both the results for liver and bone ALP are higher than those found by the modified electrophoretic method (liver ALP = 58 U/L, bone ALP = 114 U/L). Therefore, samples which contain significant amounts of intestinal ALP give falsely elevated liver and bone ALP activities by heat-inactivation. Sample 2 gave a liver ALP activity of 1033 U/L and a bone ALP activity of 0 U/L (total ALP activity = 1033 U/L). The heat-inactivation method does not distinguish between liver and high-molecular-mass ALP.

Table 2.2. Between-batch precision for liver and bone ALP measurement by heat-inactivation and agarose electrophoretic methods.

Method	Liver ALP			Bone ALP		
	Mean U/L	SD U/L	CV %	Mean U/L	SD U/L	CV %
Heat-inactivation	103	4.6	4.5	59	5.4	8.8
Agarose electrophoresis						
Neuraminidase	67	7.6	11.3	112	7.6	6.9
Lectin	57	6.6	11.6	118	8.1	6.8

2.4.2. Agarose electrophoresis

Introduction

Isoenzyme separation on 1% agarose gels was performed on precast gels using Tris-bicine as buffer. The gels were commercially available from Corning. Following electrophoresis, a substrate containing 4 methyl-umbelliferyl phosphate was added to the gels. 4-methylumbelliferone released by the action of ALP gives fluorescent bands on the gel in positions corresponding to the different ALP forms. However, when electrophoresis was carried out as described in the kit manufacturer's insert, poor separation was found between liver and bone bands. Neuraminidase treatment prior to electrophoresis (Moss and Edwards, 1984) or lectin-affinity electrophoresis (Rosalki and Foo, 1985) had to be used to obtain adequate separation and enable quantitative scanning of the gels.

Method

(i) Pretreatment with neuraminidase.

10 μl of neuraminidase (activity 500 units/ml, protein concentration 20 μg/ml) were added to 50 μl of plasma in Cobas cups and the samples incubated for 15 minutes at 37^{o}C in a water bath. After the incubation period, samples were immediately placed in ice. Samples were diluted with saline to give an approximate ALP activity of 100 U/L.

(ii) Lectin-affinity electrophoresis.

After blotting the agarose gel with filter paper, 4 ml of 50 mg/L wheat germ lectin was added to the gel and left in contact with the gel for 15 minutes. The excess solution was drained and the sample wells blotted before addition of diluted sample.

(iii) Electrophoresis.

1 μl of diluted sample was added to each well. Electrophoresis was carried out using a horizontal tank containing Tris-bicine buffer (90 mmol/L Tris, 22 mmol/L bicine, pH 9.8) for a period of 20 minutes at 170 volts. Substrate (0.5 mmol/L 4-methylumbelliferyl phosphate in 1.36 mol/L AMP buffer, 60 mmol/L $MgCl_2$ and 20% sucrose, pH 9.8) was added to the gels and these were placed in an incubator for 15 minutes at 37°C. Gels were then dried in an oven (55°C for 20 minutes) and scanned on the Corning 720 densitometer set in the fluorometric mode. All measurements were made in duplicate.

Results

Intestinal ALP, although appearing as a clearly separate peak in the unmodified method, was apparent as a shoulder on the retarded bone band with both modifications (i,ii). High-molecular-mass ALP was not separated from liver ALP on the agarose gel system. To avoid variations due to the presence of high-molecular-mass ALP and intestinal ALP, precision was checked using samples which on qualitative polyacrylamide gel electrophoresis showed only liver and bone bands. The between-batch precision for the neuraminidase-modified method and for the lectin-affinity method for liver ALP and bone ALP measurements is given in Table 2.2. Correlation between the two modifications of the agarose method was close ($r = 0.98$ for liver ALP, $r = 0.99$ for bone ALP; $n = 14$).

2.4.3. Chromatography: FPLC

Introduction

Schoenau et al, 1986, reported a method for separating ALP isoenzymes by high performance liquid chromatography using an anion exchange column ("Mono Q") and eluting with a stepwise gradient of lithium chloride (LiCl). Their chromatograms showed two distinct peaks for bone ALP and two for liver ALP.

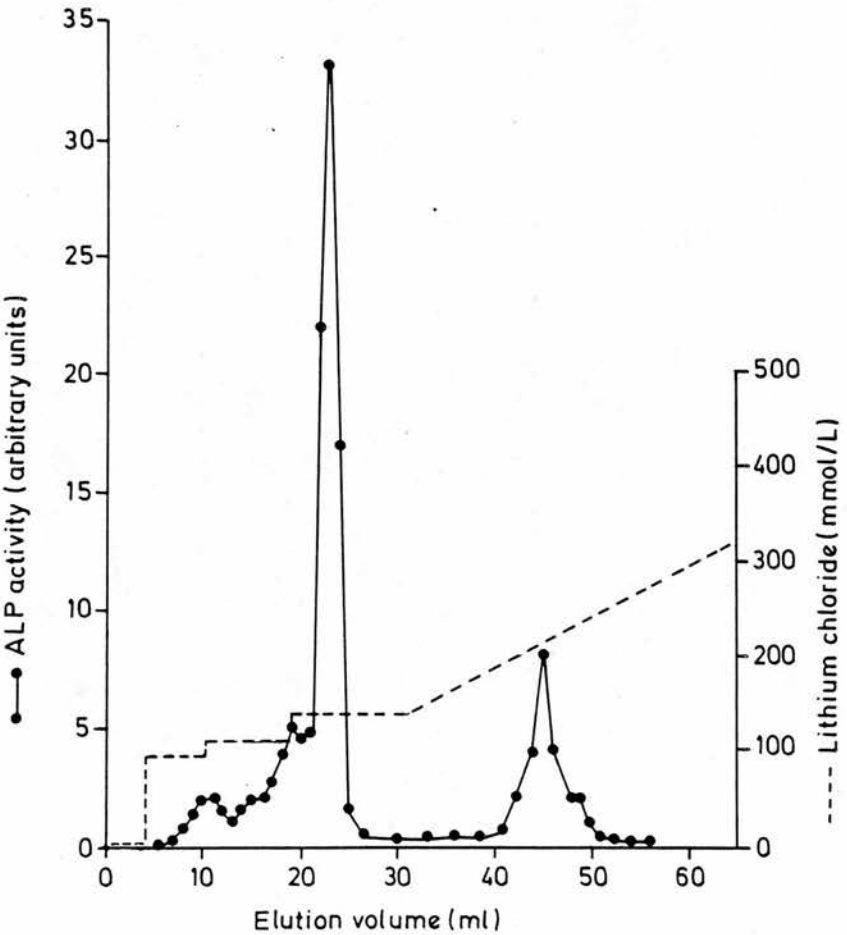
Methods and Results

The basic FPLC system (Fast Protein Liquid Chromatography) from Pharmacia was used. This consists of a gradient programmer, two precision pumps, UV monitor, fraction collector, recorder and injector. All buffers were filtered (0.22 μm filter) and degassed before use. Serum samples were filtered before use (0.22 μm sterile filter). The following gradients were used:

(i) Conditions of Schoenau et al, 1986.

The column (Mono Q HR 5/5) was equilibrated with 10 ml of 1 mol/L Tris-HCl (pH 8.2) buffer, followed by 5 ml of 20 mmol/L Tris-HCl (pH 8.2) at a flow rate of 1 ml/min. 500 μl of serum were added, and the isoenzymes eluted stepwise with LiCl in 20 mmol/L Tris-HCl buffer (4 min no added LiCl, 6 min with 90 mmol/L, 9 min with 105 mmol/L, 12 min with 130 mmol/L and then a linear gradient until a LiCl concentration of 500 mmol/L was reached). 1 ml fractions were collected and ALP activity measured on the Cobas Bio analyser. Lithium concentrations in selected fractions were measured by flame photometry and the actual lithium concentrations found shown on the chromatograms. A typical chromatogram is given in Figure 2.1. A major peak eluted at a LiCl concentration of 140 mmol/L with minor peaks eluting before this and high-molecular-mass ALP eluting at around 220 mmol/L.

Figure 2.1. Chromatogram of sample containing liver, bone and high-molecular-mass ALP. Separation using the conditions of Schoenau et al, 1986.



(ii) Linear gradient

A linear gradient of LiCl (0 to 500 mmol/L) did not separate liver and bone ALP. However, when samples were treated with neuraminidase (15 min at 37^{0}C) and the pH of the Tris-HCl buffer reduced to pH 7.0, it was possible to get separation between the two forms of ALP. Figure 2.2 shows the results obtained for a sample containing liver and bone ALP. The bone peak appears before the liver peak, both peaks eluting at a lower LiCl concentration compared to the untreated sample (Figure 2.1).

(iii) Stepwise gradient

A better separation was obtained using the conditions in (ii) above but with a stepwise gradient (5 min no added LiCl, 10 min with 35 mmol/L LiCl, 10 min with 70 mmol/L LiCl, 5 min with 175 mmol/L LiCl). Figure 2.3 shows the elution pattern of a sample containing liver and bone ALP, bone ALP eluting earlier as a clearly defined peak compared to liver ALP.

The chromatograms obtained by Schoenau et al, 1986, could not be reproduced; other workers (Gonchoroff and O'Brien, 1988) were also unable to obtain resolution of liver and bone ALP using these conditions and their chromatogram was similar to the one shown in Figure 2.1. It was possible to resolve liver and bone ALP by using neuraminidase-treated samples, reducing the pH of the buffer and using a stepwise gradient. However, the FPLC system was found to be unsuited for use with serum samples, since high back-pressures developed in the system. In addition, repeated injections of serum resulted in a deterioration in the resolving power of the column. Dilution of the sample overcame these problems but led to a loss in sensitivity.

Figure 2.2. Chromatogram of sample containing liver and bone ALP (sample was pretreated with neuraminidase; separation using a linear gradient of lithium chloride in Tris-HCl buffer, pH 7.0).

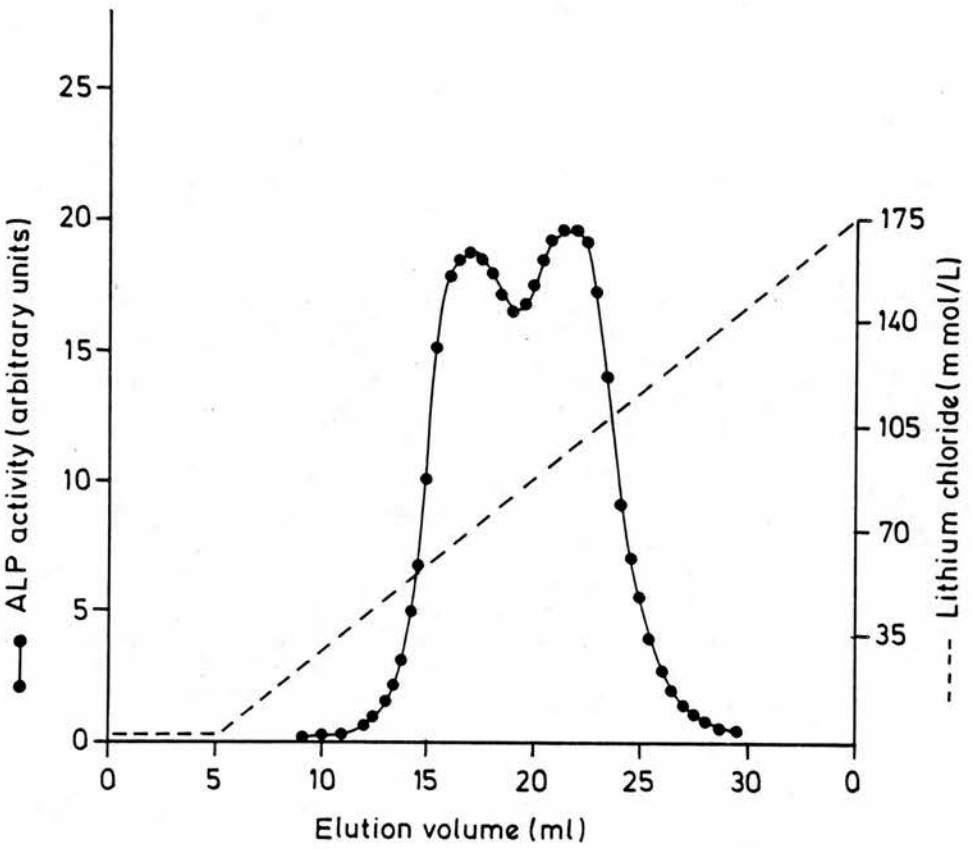
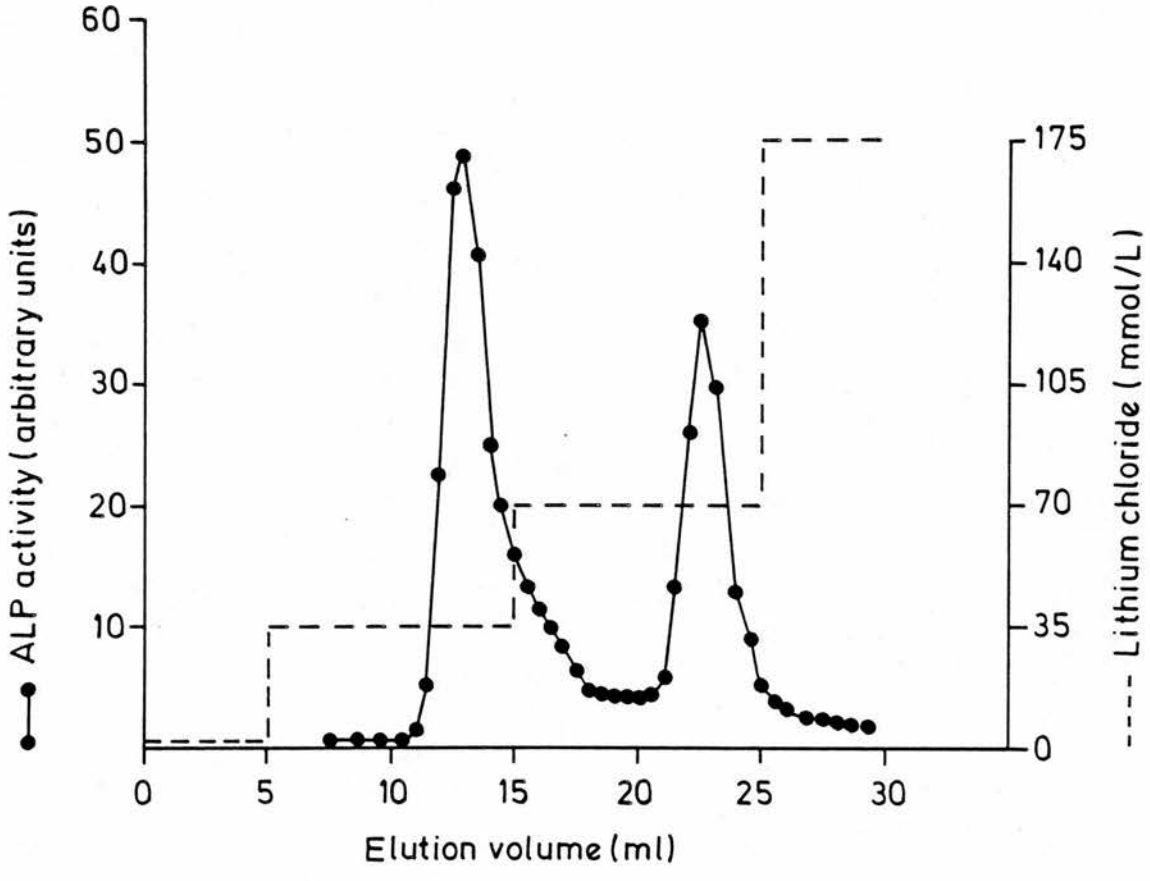


Figure 2.3. Chromatogram of sample containing liver and bone ALP (sample was pretreated with neuraminidase; separation using a stepwise gradient of lithium chloride in Tris-HCl buffer, pH 7.0).



2.4.4. Lectin-precipitation

Introduction

Addition of wheat germ lectin to plasma results in the precipitation of bone ALP, all other ALP forms remaining in the supernatant (Rosalki and Foo, 1984). Since the aim was to measure all the ALP forms individually in plasma, the lectin-precipitation method was assessed mainly for use as an independent measure of bone ALP, which could then be compared with the quantitative polyacrylamide gel method (Section 2.4.5).

Method

Cord plasma (bone band only on qualitative polyacrylamide gel electrophoresis) and plasma samples with only the liver band on electrophoresis were used. All other plasma samples contained varying proportions of liver and bone ALP with minimal amounts of other ALP forms on electrophoresis. Samples were set up, in duplicate, as follows: 50 μl of plasma were incubated with 50 μl of 5 g/L wheat germ lectin solution in Cobas cups for 30 minutes at 37°C (Behr and Barnert, 1986). Samples were centrifuged for 15 minutes at 2000 xg, the supernatant removed to a fresh cup and the precipitate solubilised in 100 μl of 154 mmol/L sodium chloride. Alkaline phosphatase activities in the supernatant and solubilised precipitate were measured on the Cobas Bio as described for total ALP.

Results and Assessment

1.4% of total ALP activity of cord plasma samples (n = 2) remained in the supernatant; 5.3% of total ALP activity of samples containing only liver ALP (n = 2) was found in the solubilised precipitate. Therefore, ALP activity of the solubilised precipitate was taken as an approximate measure of bone ALP; ALP activity of the supernatant was taken as an approximate measure of liver ALP. Within-batch precision for supernatant ALP measurement was 1.3%; for solubilised precipitate ALP measurement it was 8.4%. Insufficient data was available for assessment of between-batch precision.

Mean percentage recovery {[(supernatant ALP + precipitate ALP) / total ALP] x 100} of ALP activity in samples was 102% (range 95.5 to 113.3%). ALP activity of solubilised precipitate was approximately equal to (total ALP - supernatant ALP) in most samples. Because of problems encountered in solubilising the precipitate and the poor precision

obtained for the measurement of ALP activity of the precipitate, bone ALP was calculated from (total ALP - supernatant ALP) for the comparison studies given below.

2.4.5. Polyacrylamide gel electrophoresis

A. Qualitative polyacrylamide gel electrophoresis

Introduction

Liver, bone and intestinal ALP can be separated by 7% acrylamide gel; high-molecular-mass ALP remains at the origin. The polyacrylamide gel method used was a modification of the method of Kaplan and Rogers, 1969.

Method

Gel slabs (1.5 mm thickness) were prepared by mixing together 20 ml 14% acrylamide, 20 ml buffer (0.4 mol/L Tris-borate, 0.5 mmol/L $MgCl_2$, pH 9.5) and 0.2 ml TEMED. Ammonium persulphate (35 mg) was then added, the solution was mixed and poured immediately between two glass plates set up in the Gel Casting Unit (LKB). The well former was placed in position. Once the gel had set, the well former was removed; the sample wells were washed with distilled water and then with buffer and the glass plates, with formed gel, placed in the tank.

Samples were diluted with 20% sucrose to give an approximate ALP activity of 50 to 100 U/L. 20 μl of diluted sample were added to each well. A marker (whole blood diluted in sucrose) was added to the end well. Electrophoresis was carried out at a constant voltage of 300 volts (initial current of 50 milliamps) in a water-cooled vertical electrophoresis tank using Tris-borate as buffer. The gel was run until the marker had migrated 7 cm (approximately 3 hours). On this system, it was possible to run 2 gels at the same time with 10 samples on each gel.

After electrophoresis, gels were placed in substrate/dye solution (0.75 mmol/L α-naphthyl acid phosphate and 0.5 g/L 4-aminodiphenylamine diazonium sulphate in Tris-borate buffer) and left overnight at 4°C. Gels were cleared using 7% acetic acid and then stored in plastic bags. All gels were assessed qualitatively by giving each band of ALP activity a score which depended on the intensity of the band.

Results

Figure 2.4 shows the separation of the main ALP forms present in plasma on 7% acrylamide gels. Sample 1 (track 1a) shows liver ALP and high-molecular-mass ALP, which remains at the origin. Sample 2 (track 2a) is cord plasma and shows the position of bone ALP. Sample 3 (track 3a) contains liver and bone ALP; sample 4 (track 4a) contains liver, bone and intestinal ALP.

The separation on polyacrylamide gel electrophoresis was adequate for the qualitative assessment of isoenzyme patterns and was used throughout to determine the main ALP forms present and to look for rarer forms of ALP such as ALP-immunoglobulin complexes and cancer-associated isoenzymes (Section 2.7.2).

B. Quantitative polyacrylamide gel electrophoresis

Introduction

The method described in A was modified by treating samples with neuraminidase (Moss and Edwards, 1984) prior to electrophoresis to enhance the separation between liver and bone ALP so that the bands could be scanned to give quantitative results. As a result of neuraminidase treatment, the retarded bone band moved in the same position as the intestinal band (which is unaffected by neuraminidase). Ways of removing the interference of intestinal ALP in bone ALP measurement were investigated.

Methods and Results

(i) Treatment with neuraminidase.

Neuraminidase treatment was carried out as described previously. Figure 2.4 shows the effect on the mobility of liver and bone ALP when samples containing these forms were treated with neuraminidase (tracks 1a and 1b for liver ALP, tracks 2a and 2b for bone ALP). When a sample containing both liver and bone ALP was treated with neuraminidase (track 3b), clearly separate bands for liver and bone ALP were obtained. Tracks 4a and 4b show how, after neuraminidase treatment, the retarded bone band moves in the same position as the intestinal band.

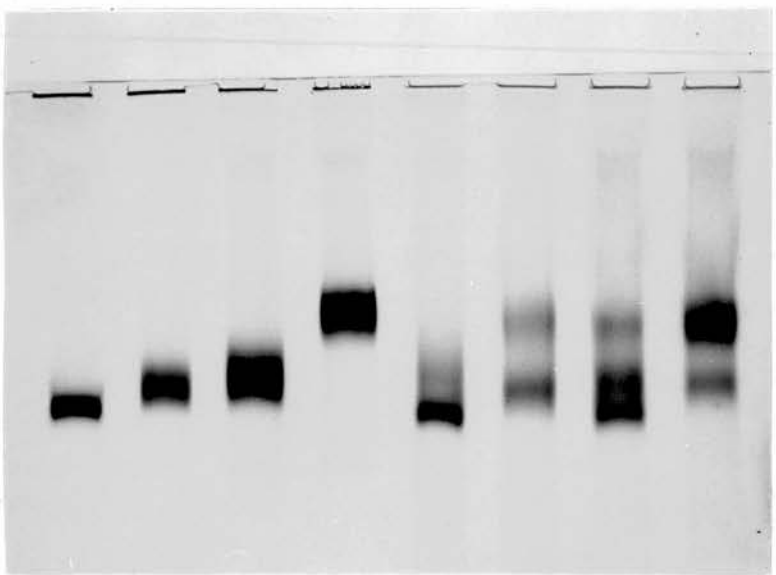
Figure 2.4. Separation on 7% polyacrylamide gel. Samples (a) unmodified and (b) treated with neuraminidase.

Sample 1: liver and high-molecular-mass ALP.

Sample 2: bone ALP.

Sample 3: liver and bone ALP.

Sample 4: liver, bone and intestinal ALP.



(ii) Ways of overcoming intestinal ALP interference in bone ALP measurement.

(a) Incubation of gels with L-phenylalanine.

Increasing amounts of a sample containing mainly bone ALP were added to a sample with mainly liver and intestinal bands present on electrophoresis (Figure 2.5. Gels I and II. Track 1a, no added bone ALP; track 2a, 25 μl added bone ALP; track 3a, 50 μl added bone ALP; track 4a, 100 μl of bone ALP). Samples in tracks 1b - 4b were the same as in tracks 1a - 4a except that these had been treated with neuraminidase prior to electrophoresis. Following electrophoresis, gel I was stained as described in A above while gel II was stained in the presence of 10 mmol/L L-phenylalanine. Incubation of the gel with L-phenylalanine resulted in the removal of the intestinal band (compare gel I, track 1a with the corresponding track on gel II). When samples were treated with neuraminidase, L-phenylalanine reduced the interference of intestinal ALP in bone ALP quantitation since in all cases the "bone bands" in gel II (tracks 1b, 2b, 3b, 4b) were fainter than the corresponding tracks in gel I, where the band was made up of bone and intestinal ALP.

Although it is not possible to relate conditions in the gel directly to those in an aqueous solution, the behaviour of intestinal ALP towards L-phenylalanine in solution was investigated to see whether inhibition of intestinal ALP, at least in solution, was complete. Alkaline phosphatase activities of samples containing only liver or bone ALP on electrophoresis and of an extract from human small intestinal mucosa were measured in the absence and presence of 10 mmol/L L-phenylalanine using α-naphthyl acid phosphate as substrate. Substrate, buffer and Mg^{2+} ion concentrations and the buffer pH were the same as those used for staining the gels. Measurements were made on the Cobas Bio at a wavelength of 340 nm.

Mean percentage inhibition of liver or bone ALP by 10 mmol/L L-phenylalanine was 35.6% (SD 2.1%, range 33 - 38%, n = 4); for the intestinal extract it was 81.5%. Therefore, in aqueous solution, 10 mmol/L L-phenylalanine does not inhibit intestinal ALP totally and it causes some inhibition of liver and bone ALP. Other concentrations of L-phenylalanine were tried but did not give better discrimination in terms of inhibition of ALP forms. The use of L-phenylalanine was therefore abandoned and a more specific way of removing intestinal ALP was sought.

Figure 2.5. Incubation of polyacrylamide gel without (gel I) and with (gel II) 10 mmol/L L-phenylalanine. See text for details.

GEL I

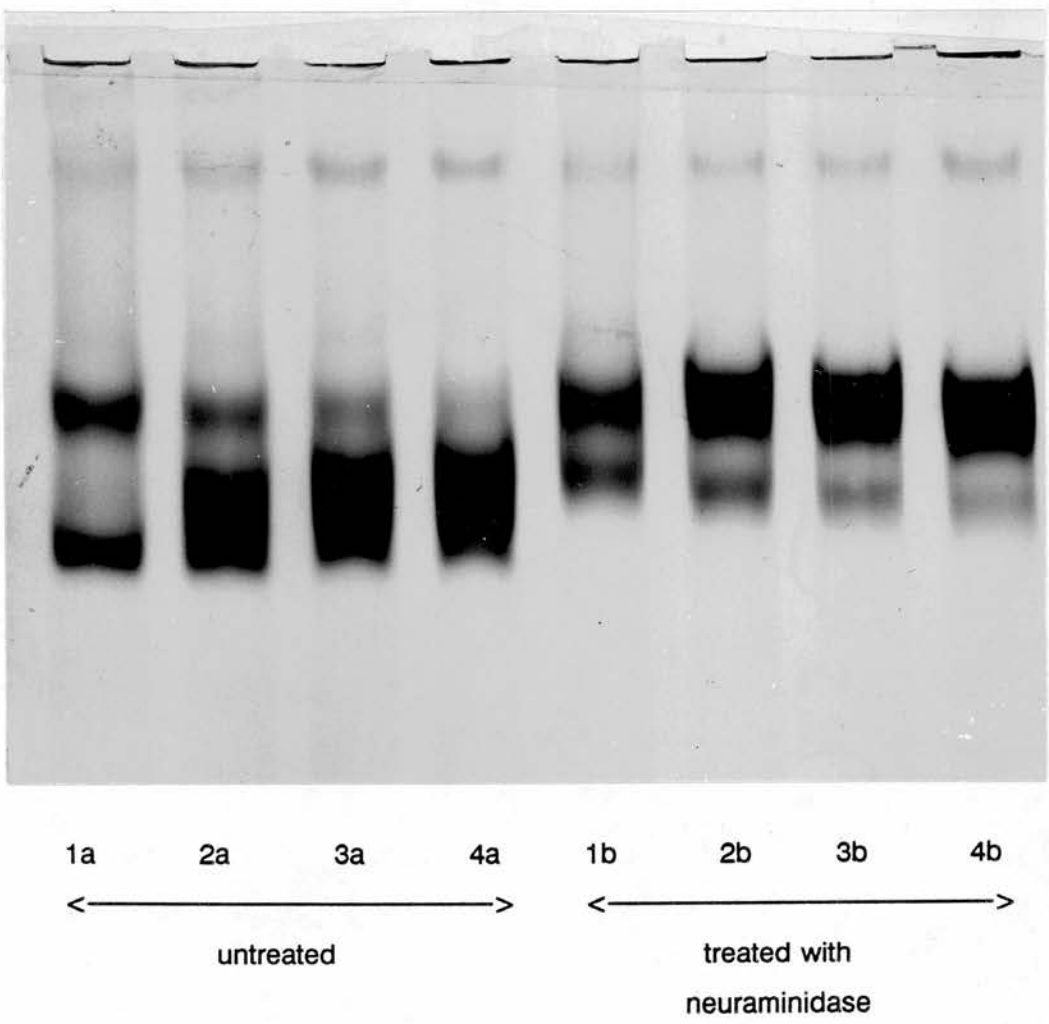
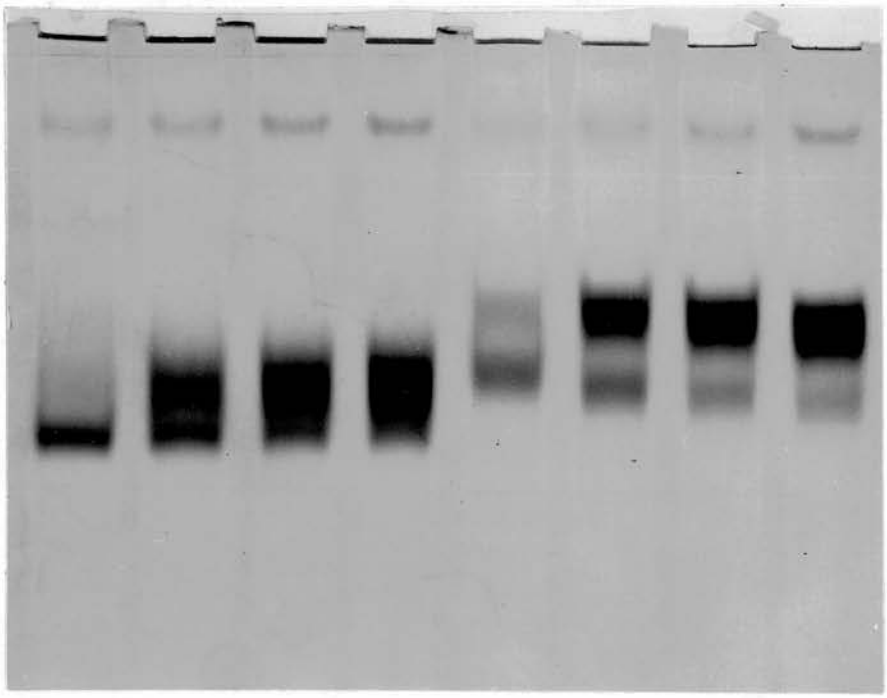


Figure 2.5. (Cont.)

GEL II (with 10 mmol/L L-phenylalanine)



(b) Monoclonal antibody to intestinal ALP

A monoclonal antibody to intestinal ALP (AAP-1) was added to plasma samples at final dilutions of 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600 and 1:3200. Electrophoresis and staining of the gels was carried out as described in Section 2.4.5 (A). Figure 2.6 shows the results obtained; the intestinal band is complexed and removed by the monoclonal antibody (tracks 2 - 5) and appears again at higher dilutions of the antibody (tracks 6 - 8). A final dilution of antibody in plasma of 1:200 was chosen (track 4) and used in all experiments. The antibody had no effect on the mobility of the liver band (Figure 2.6) or the bone band (results not shown). There was a very slight increase (approximately 3 U/L) in total ALP activity on addition of the antibody to plasma at this dilution.

(iii) Summary of the modified electrophoretic method.

(a) 5 μl of 1:20 dilution in distilled water of monoclonal antibody to intestinal ALP were added to 45 μl of plasma in Cobas cups.

(b) 10 μl of neuraminidase were added to the samples and these were incubated for 15 minutes at 37^{0}C in a water bath. After the incubation period, samples were placed on ice.

(c) Electrophoresis and staining of the gels was carried out as described in Section 2.4.5 (A). All samples for quantitative liver and bone ALP measurement were set up in duplicate.

(d) Gels in plastic bags were scanned on the Appraise scanner (set in absorbance mode, filter 520 nm). Only the liver and bone bands were scanned. The scanner gives percentage area under each peak; this was expressed in U/L by multiplying values obtained for each peak by (total ALP - intestinal ALP - high-molecular-mass ALP). The values for intestinal ALP and high-molecular-mass ALP were obtained as described in Sections 2.5 and 2.6 respectively. Figure 2.7 gives a typical separation obtained for a sample containing liver, bone and intestinal ALP with a densitometric scan of the liver and bone bands.

Figure 2.6. Monoclonal antibody to intestinal ALP added at increasing dilutions to a plasma sample containing liver and intestinal ALP.
(Track 1 - unmodified sample; tracks 2 to 8 - dilutions of antibody:plasma from 1:50 to 1:3200).

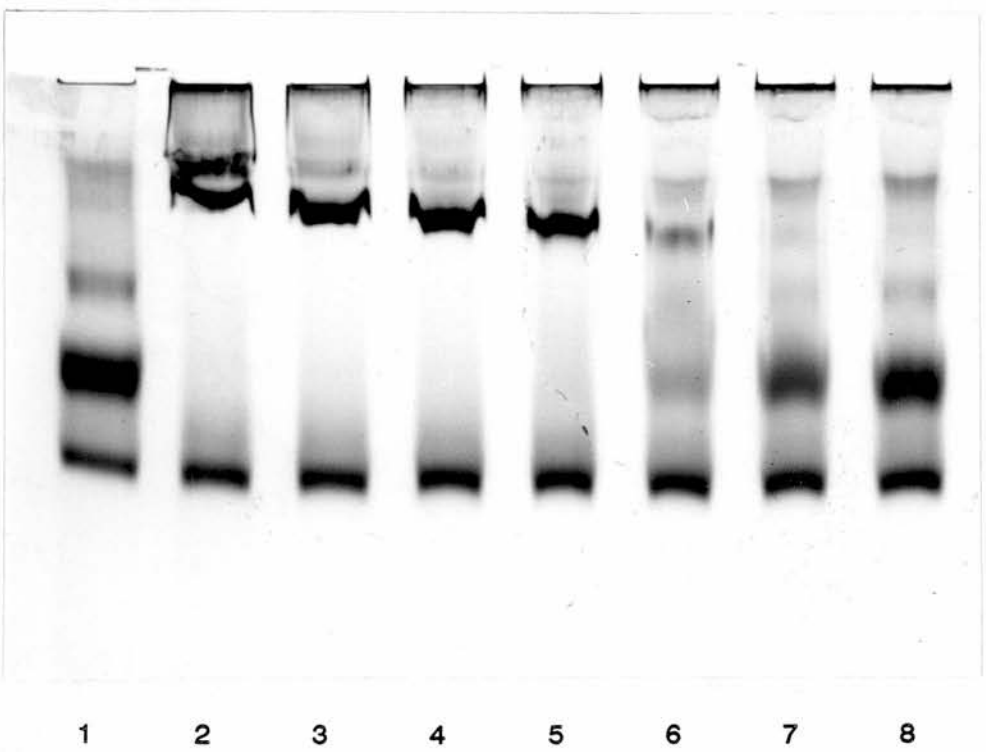
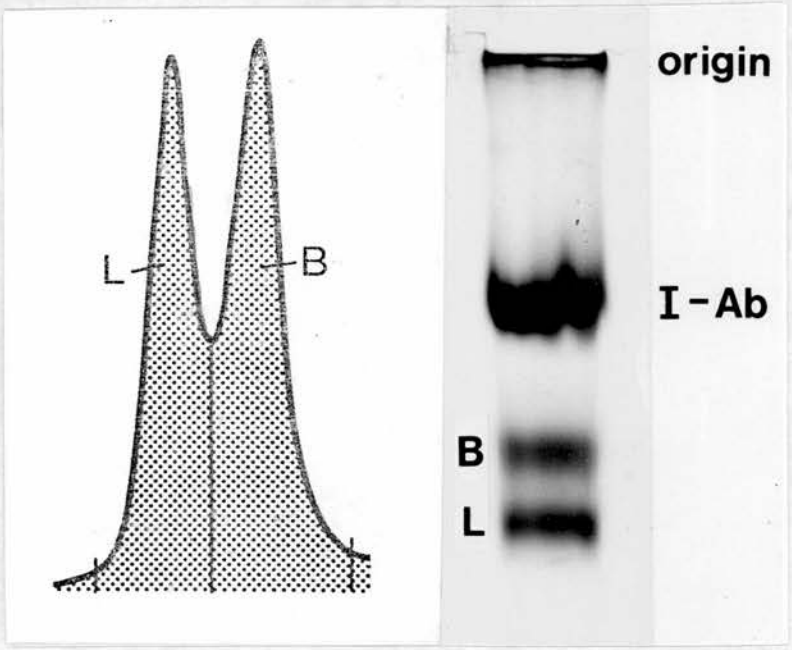


Figure 2.7. Separation of a sample containing liver, bone and intestinal ALP using the modified electrophoretic method (see text for details).
Scan of the liver (L) and bone (B) bands and the position of intestinal ALP-antibody complex (I-Ab) is shown.



(iv) Comparison with other methods.

Samples containing only liver and bone bands on electrophoresis were used in the comparison studies. The modified electrophoretic method was compared to the heat-inactivation method and to the lectin-precipitation method. All measurements were made in duplicate. Figure 2.8 shows the correlation between the modified electrophoretic method and heat-inactivation method for liver ALP [Figure 2.8a; slope = 1.19 (SE = 0.13); intercept = -25.6 U/L (SE = 13.8); r = 0.91] and for bone ALP [Figure 2.8b; slope = 1.15 (SE = 0.06); intercept = -9.4 U/L (SE = 7.9); r = 0.98]. Comparisons between the modified electrophoretic method and the lectin-precipitation method are given in Figure 2.9 [Figure 2.9a; liver ALP measurement; slope = 1.09 (SE = 0.08); intercept = -14.2 U/L (SE = 9.2); r = 0.98. Figure 2.9b; bone ALP measurement; slope = 1.02 (SE = 0.04); intercept = -3.7 U/L (SE = 4.0); r = 0.99].

(v) Precision.

The between-batch precision (CV) for liver and bone ALP measurement by the modified electrophoretic method assessed for two ranges (liver/bone ALP <100 U/L and liver/bone ALP >100 U/L) is presented in Table 2.3. Two plasma samples (1 and 2), with strong intestinal bands on electrophoresis, were run on different gels; the between-batch precision for liver and bone ALP measurement for these samples is given in Table 2.4.

Table 2.3. Between-batch precision for liver and bone ALP measurement.

	n	Range U/L	Mean U/L	SD U/L	CV %
Liver ALP	40	<100	40	2.2	5.5
	6	>100	255	6.3	2.5
Bone ALP	40	<100	47	3.5	7.4
	6	>100	123	4.1	3.3

Figure 2.8a. Correlation between the modified electrophoretic method (eph) and heat-inactivation method (heat) for the measurement of liver ALP.

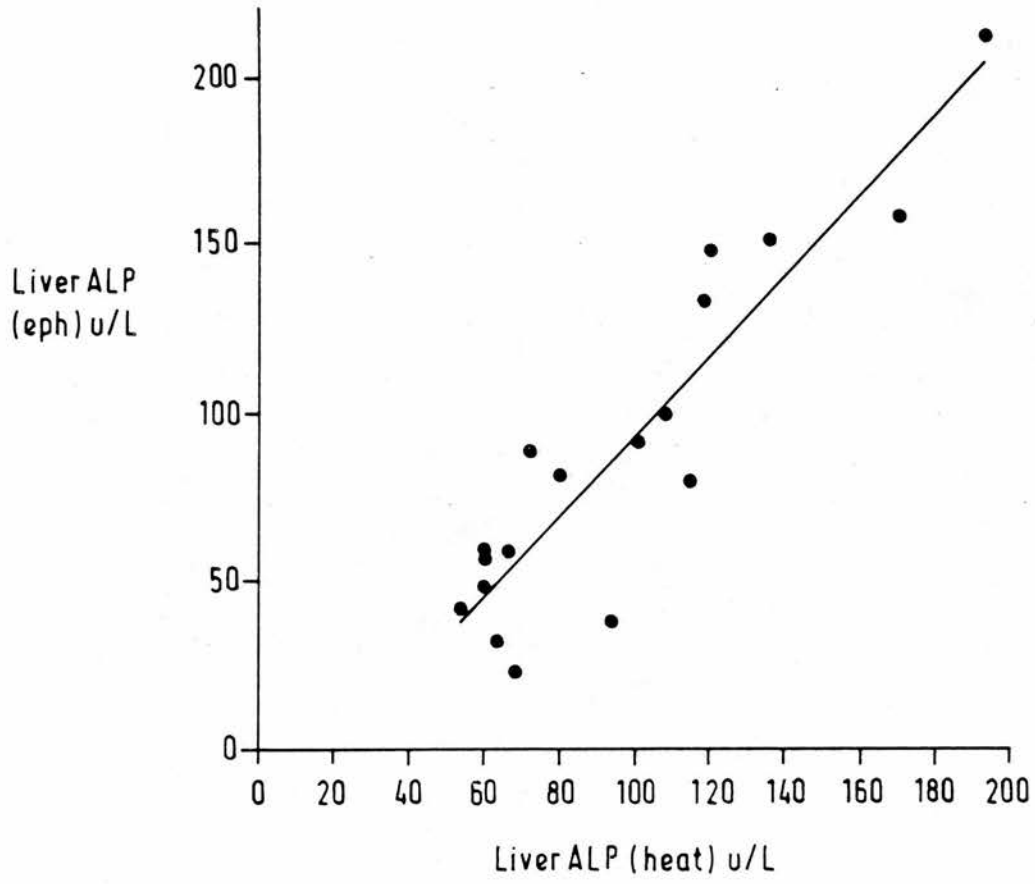


Figure 2.8b. Correlation between the modified electrophoretic method (eph) and heat-inactivation method (heat) for the measurement of bone ALP.

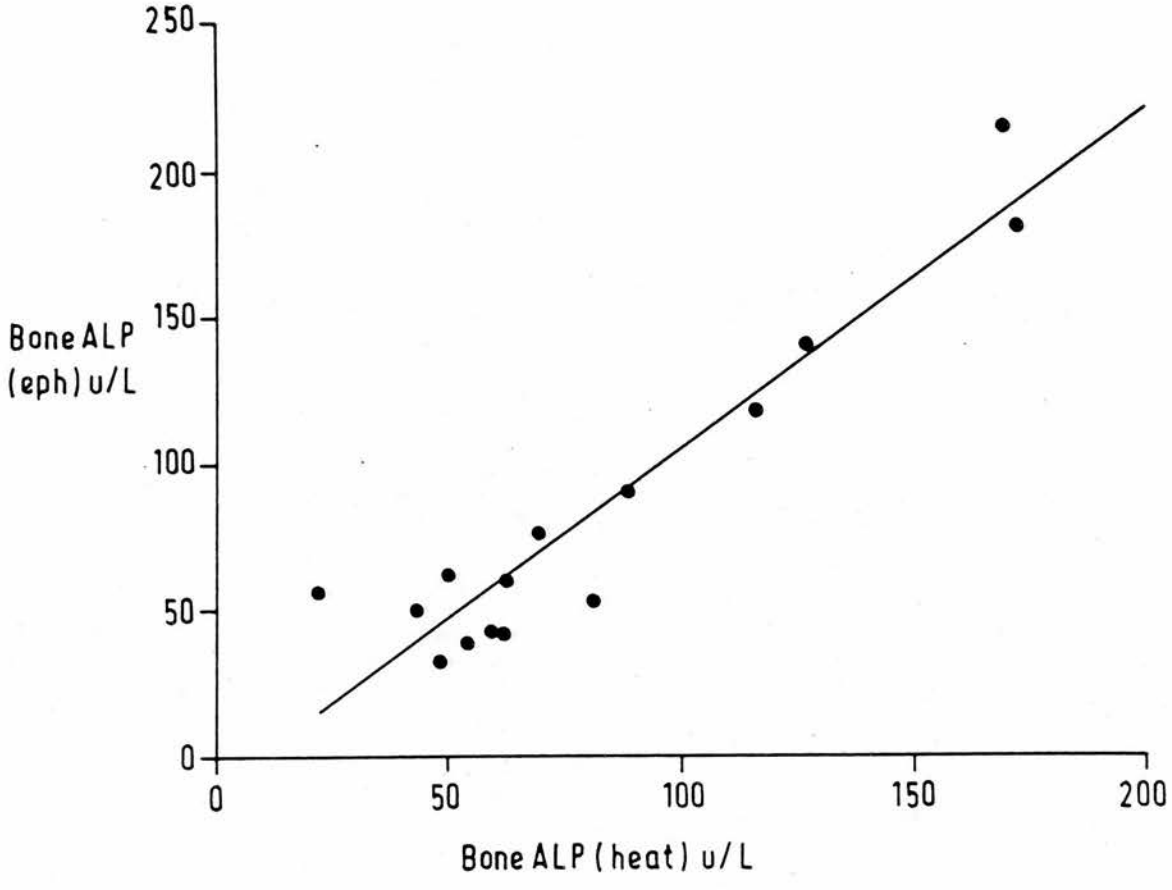


Figure 2.9a. Correlation between the modified electrophoretic method (eph) and the lectin-precipitation method (lectin) for the measurement of liver ALP.

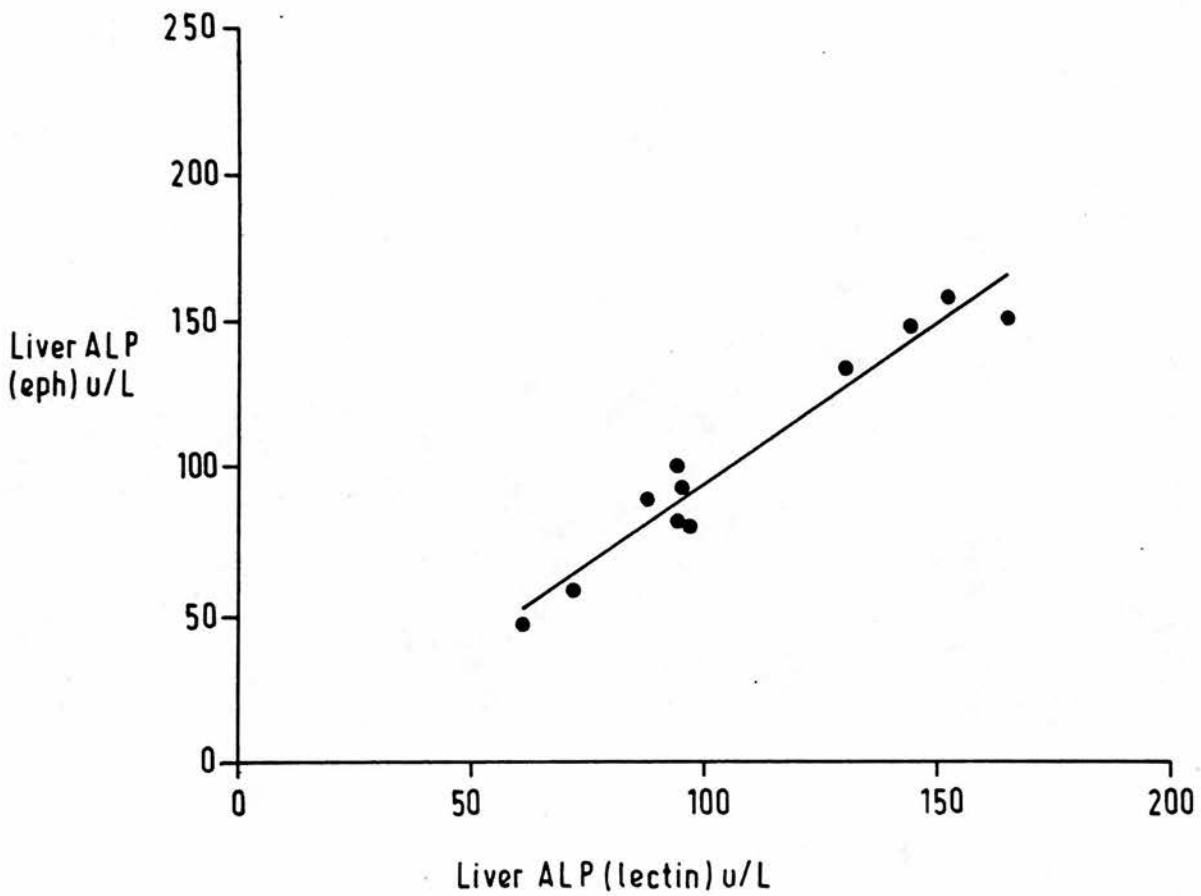


Figure 2.9b. Correlation between the modified electrophoretic method (eph) and the lectin-precipitation method (lectin) for measuring bone ALP.

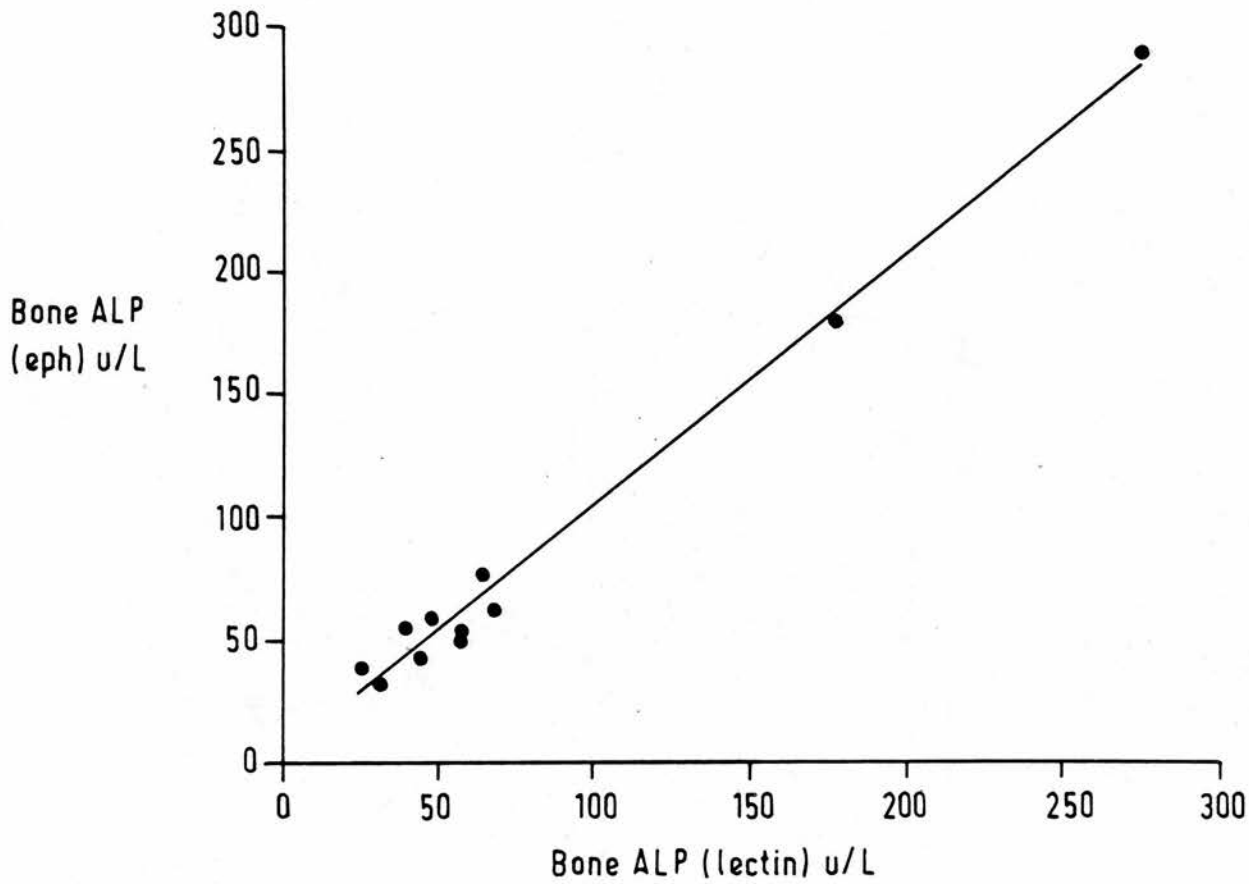


Table 2.4. Precision for liver and bone measurements for two samples containing strong intestinal bands.

		Liver ALP			Bone ALP		
	n	Mean U/L	SD U/L	CV %	Mean U/L	SD U/L	CV %
Sample 1	7	73	3.2	4.3	20	1.6	8.1
Sample 2	9	12	1.8	14.6	109	3.6	3.3

(vi) Linearity.

The linearity of the method for bone ALP was checked by adding cord plasma, containing only bone ALP, to a plasma sample and was found to be linear up to a measured bone ALP of at least 100 U/L.

(vii) Interference.

A raised bone ALP was shown not to interfere in the quantitation of liver ALP by measuring liver and bone ALP in plasma samples from children. Mean bone ALP, which is physiologically raised in children, was 162 U/L (SD = 66). Mean liver ALP was 28 U/L (SD = 19). This was not different to the activity found in blood donors (mean liver ALP = 29 U/L, SD = 13; Chapter 5) indicating that the interference in liver ALP measurement by bone ALP is negligible.

Bone and liver ALP were measured in plasma from 85 patients with liver disease. Mean liver ALP was 275 U/L (SD = 333 U/L). Mean plasma bone ALP was 39 U/L (SD = 37 U/L); this was not significantly different to the mean bone ALP of blood donors (mean bone ALP = 33 U/L, SD = 12 U/L; Chapter 5). These results suggest that a raised liver ALP does not interfere in bone ALP measurement.

Discussion

The modified electrophoretic method gave results which compared well with heat-inactivation (Figures 2.8a, 2.8b) and even more closely with lectin-precipitation (Figures 2.9a, 2.9b). Interference by intestinal ALP was overcome by the addition of a monoclonal antibody to intestinal ALP. High-molecular-mass ALP remains at the origin and will, therefore, not interfere in the quantitation of the liver and bone bands. The method gave fairly good precision even for samples which contained intestinal bands (Table 2.4). The method, although time-consuming, is suitable for the measurement of liver and bone ALP in large numbers of samples.

Both intestinal and high-molecular-mass ALP interfered in the measurement of liver and bone ALP by the heat-inactivation method (Section 2.4.1) and the agarose method (Section 2.4.2). These two methods were, in general, less precise than the modified electrophoretic method (compare Table 2.2 with Table 2.3).

In terms of ease of performance, the agarose method was the least labour-intensive and the least time-consuming. However, some gels showed high background fluorescence which resulted in poor resolution. This meant that these gels had to be repeated. The commercial agarose gels were also more expensive than the polyacrylamide gels.

The heat-inactivation method was labour-intensive and required great care in temperature and time control. Preliminary electrophoresis of samples prior to quantitation by heat-inactivation was necessary so that the presence of significant amounts of other ALP forms could be recognised. This means that there is no advantage in the use of heat-inactivation compared to electrophoretic methods. Problems with the FPLC method (Section 2.4.3) have already been discussed. The main drawback of FPLC was its unsuitability for measurements in large numbers of serum samples. Because the lectin-precipitation method is rapid and easy to carry out, it is useful in situations where bone ALP measurement alone is required.

In conclusion, the modified electrophoretic method was found to be the most suitable of the methods discussed above in terms of accuracy, precision and suitability for large sample numbers and was used for the measurement of liver and bone ALP in the clinical studies presented in this thesis.

2.5. MEASUREMENT OF INTESTINAL ALKALINE PHOSPHATASE

Several methods for quantitation of intestinal ALP in plasma were investigated. These included the following:

(a) electrophoretic separation on agarose and polyacrylamide gels with scanning of the intestinal band.
(b) separation of intestinal ALP by FPLC ("Mono Q" column).
(c) a method based on selective inhibition.
(d) immunological methods.

The presence of aggregates of intestinal ALP gave several bands on polyacrylamide gel electrophoresis (Figure 2.6, track 1) making it difficult to accurately quantitate intestinal ALP by scanning while,on the Corning agarose electrophoretic method, intestinal ALP gave a single band which could be quantified. However, as discussed previously (Section 2.4), poor resolution because of high background fluorescence made the agarose method unreliable. Intestinal ALP was separated from liver/bone ALP by FPLC using a linear gradient of LiCl. On this gradient, intestinal ALP eluted as a single peak before the liver/bone peak at a LiCl concentration of 110 mmol/L. However, as mentioned in Section 2.4, FPLC was found to be unsuitable for the analysis of large numbers of samples. Three methods for measuring intestinal ALP were investigated more fully and will be described in detail below. These were bromotetramisole-inhibition, an immunoprecipitation method (using a monoclonal antibody to liver/bone ALP) and an enzyme-linked immunosorbent assay (ELISA).

2.5.1. Differential inhibition with bromotetramisole

Introduction

Van Belle et al, 1977, have described a method for measuring intestinal or placental ALP in serum using L-p-bromotetramisole (BT). The method is based on the finding that, at a concentration of 5×10^{-5} mol/L, BT causes approximately 90% inhibition of liver and bone ALP while inhibiting intestinal ALP by only 10%.

Methods and Results

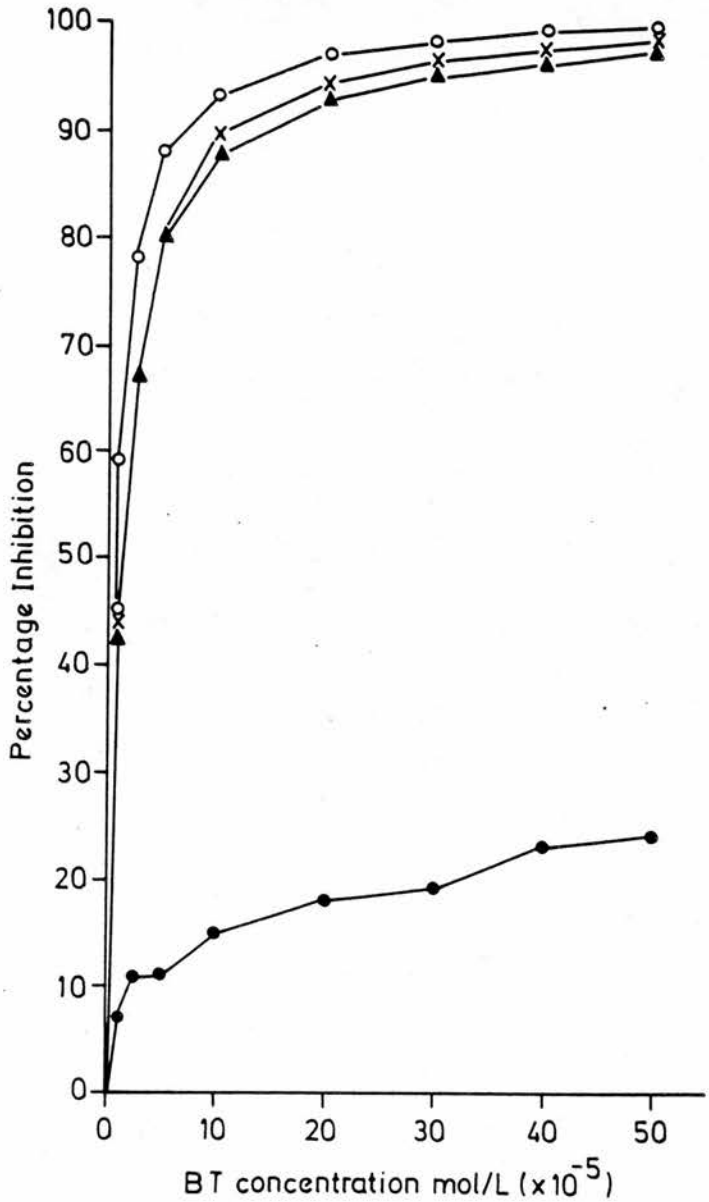
(i) Preparation of intestinal and liver extracts.

A piece of human small intestine was obtained at autopsy, washed with water and the mucosal cells scraped into 20% butanol in 0.01 mol/L Tris-HCl, pH 7.4 (McKenna et al, 1979). The mucosal cell mixture was stirred for 2 hours at 4°C and then centrifuged (1500 xg for 30 minutes). The top butanol layer was discarded, the bottom aqueous layer was divided into aliquots and these were kept at -20°C. Human liver microsome preparation was kindly provided by Dr L. McLellan. This had been prepared as described by Wolf et al, 1984. The liver microsome preparation was treated as described for the intestinal mucosal cell mixture.

(ii) Selection of appropriate BT concentration.

Plasma samples from patients with primary biliary cirrhosis (n = 2 - predominant liver band on electrophoresis) and with bone disease (Paget's disease, n = 2; prostatic carcinoma with bone metastases, n = 2 - predominant bone bands) were used. These plasma samples were diluted to give a total ALP activity of less than 500 U/L. Activities of the intestinal extract, liver microsomal extract and plasma samples were measured on the Cobas Bio using reagents and conditions for total ALP measurement (Section 2.3). Varying amounts of a stock solution of 5×10^{-3} mol/L BT were then added to the ALP reagent to give concentrations of BT ranging from 1 to 50×10^{-5}mol/L. Alkaline phosphatase activities in the presence of BT ("BT-inhibited ALP") were measured in duplicate at each concentration of BT. Values for percentage inhibition at the different concentrations of BT for the intestinal extract (total ALP activity = 575 U/L), for the liver microsomal extract (total ALP activity = 179 U/L) and for the plasma samples with predominant liver or bone bands were plotted against BT concentration (Figure 2.10). Plasma samples with liver or bone bands behaved in the same way towards BT; their inhibition pattern with BT was similar to that of the liver extract. A concentration of BT of 5×10^{-5} mol/L BT was chosen since this gave nearly maximal difference between the intestinal and liver extracts. It was also the concentration used by Van Belle et al, 1977. Repeat analyses (n = 6) at a concentration of 5×10^{-5} mol/L BT gave mean percentage inhibition of 13.54% (SD 0.95) for the intestinal extract and 86.07 (SD 0.23) for the liver extract.

Figure 2.10. Percentage inhibition of ALP against bromotetramisole (BT) concentration. Percentage inhibition equal to [(Total ALP - "BT-inhibited ALP") / total ALP] x 100. Samples as follows:
Intestinal extract (-●-●-); liver extract (-o-o-); plasma samples [n = 2] with liver bands (x-x-x); plasma samples [n = 4] with bone bands (-▲-▲-).



Intestinal ALP was calculated using a formula derived from the total and BT-inhibited (at a concentration of 5×10^{-5} mol/L) ALP activities (expressed in U/L).

A_0 = Total ALP activity

A_1 = BT-inhibited ALP activity

I = Intestinal ALP activity

L = Liver (and bone ALP) activity

Then:

$$I + L = A_0 \qquad (1)$$

$$0.865I + 0.14L = A_1 \qquad (2)$$

Eliminating L from (2):

$$0.865I + 0.14 (A_0 - I) = A_1$$

Solving this equation for I gives:

$$I = (A_1 - 0.14\ A_0) / 0.725$$

(iii) Precision.

The between-batch coefficient of variation for the derived value for intestinal ALP was assessed by repeat analysis in different runs of specimens from patients and was found to be 6.1% (range 3 - 44 U/L, n = 13).

2.5.2. Immunoprecipitation

Introduction

A monoclonal antibody to liver/bone ALP (TRA 254/2) when added to plasma forms soluble complexes with liver and bone ALP. A solid phased sheep anti-mouse immunoglobulin attaches to the complexed liver/bone ALP. After centrifugation, intestinal ALP will be left in the supernatant and can be measured.

Methods and Results

(i) Optimisation of monoclonal antibody concentration.

Monoclonal antibody to liver/bone ALP was added to plasma samples with predominant liver or bone bands on electrophoresis at final dilutions of 1:2, 1:5, 1:10, 1:20, 1:40, 1:80, 1:160 and 1:320 (antibody:plasma). Polyacrylamide gel

electrophoresis was carried out. The gel photograph for a plasma sample with predominant bone band is shown in Figure 2.11. The bone band is complexed and retarded by the antibody to liver/bone ALP (tracks 2 to 6) and appears again at higher dilutions of the antibody (tracks 7 to 9). Similar results were obtained for a plasma with a predominant liver band on electrophoresis. A dilution of 1:10 of antibody was chosen (track 4); addition of antibody at this dilution caused no change in total ALP activity.

(ii) Optimisation of Sephacryl S-500-linked sheep anti-mouse immunoglobulin concentration.

Sephacryl S-500-linked sheep anti-mouse immunoglobulin was kindly provided by Dr. C. Sturgeon. This had been prepared as described by Wright and Hunter, 1982. Monoclonal antibody to liver/bone ALP was added to a plasma containing an intestinal band (1:10 antibody:plasma). The sample was then mixed with an equal volume of Sephacryl S-500-linked sheep anti-mouse immunoglobulin at various dilutions in 0.4 mol/L Tris-borate buffer, pH 9.5 (1:2, 1:4, 1:8, 1:16 and 1:32). The samples were mixed occasionally over a period of 1 hour at room temperature, centrifuged at 3000 xg for 10 minutes and ALP activities measured in the supernatants. Activities in the supernatants of the 1:2 and 1:4 dilutions of S-500-linked immunoglobulin were approximately the same. Activities in the supernatants of the other S-500 dilutions (1:8 to 1:32) increased until in the 1:32 dilution the activity was the same as that without added S-500-linked immunoglobulin. A 1:2 dilution of S-500-linked immunoglobulin was chosen for use in all experiments.

(iii) Measurement of intestinal ALP activity.

Four plasma samples with intestinal ALP bands were selected. Each sample was set up, in duplicate, as follows:

a. 100 μl of plasma (without antibody).

b. 100 μl of plasma with antibody to liver/bone ALP.

Samples were mixed with 100 μl of 1:2 dilution of S-500-linked immunoglobulin and left for 1 hour as described above. Supernatant ALP activity was measured after centrifugation in (a) and (b). Intestinal ALP activity (U/L) was calculated from [(activity of supernatant from b)/(activity of supernatant from a)] x total ALP. Figure 2.12 shows polyacrylamide gel electrophoresis of supernatants from a (without antibody) and from b (with antibody) for the four plasma samples (calculated intestinal ALP activities for

samples 1 to 4 = 23 U/L, 20 U/L, 77 U/L and 68 U/L respectively). In all cases, only the intestinal band remained in the supernatant of the sample with added antibody to liver/bone ALP.

(iv) Precision.
Within-batch precision of measurements of supernatant ALP activity of samples (without added antibody) was 1.7%. For samples with added antibody, it was 2.2%.

(v) Comparison with the BT method.
Intestinal ALP activities in 10 plasma samples, all with intestinal bands on electrophoresis, were measured by immunoprecipitation and by the bromotetramisole (BT) method. The correlation coefficient was 0.98 (x = immunoprecipitation method, y = BT method; slope = 1.06, SE = 0.08; intercept = 4.0 U/L, SE = 2.3).

2.5.3. Enzyme-linked immunosorbent assay (ELISA)

Method
The method used was a modification of that of Brock et al, 1984. Microtitre plates were coated for 2 hours with a rabbit anti-mouse immunoglobulin diluted 1:1000 in 0.05 mol/L carbonate buffer, pH 9.6. The plates were then washed three times with phosphate-buffered saline, 0.5 mol/L (pH 7.2) containing 0.5% Tween-20. Monoclonal antibody to intestinal ALP (1:1000 in phosphate-buffered saline) was added to all wells. After 2 hours, plates were washed, as before. Standard and plasma samples (diluted 1:5) were added to wells in triplicate. After an overnight incubation at room temperature, the plates were washed, incubated for 1 hour with substrate (5 mmol/L p-nitrophenyl phosphate in 1 mol/L diethanolamine, pH 9.8, containing 0.5 mmol/L $MgCl_2$), after which the reaction was stopped with 2 mol/L sodium hydroxide. Absorbance readings were made at 405 nm on an automatic plate reader. An extract of human small intestinal mucosa (prepared as described above) diluted in heat-inactivated plasma, was used to obtain a standard curve. ALP activity of the intestinal extract (measured on the Cobas Bio) was related to the absorbance readings obtained for the standard wells on the plate so that intestinal ALP activity of plasma samples could be expressed in U/L.

Figure 2.11. Monoclonal antibody to liver/bone ALP added at increasing dilutions to a plasma sample containing bone ALP.
Track 1 - unmodified sample; tracks 2 to 9 - dilutions of antibody:plasma from 1:2, 1:5 and then doubling dilutions to 1:320).

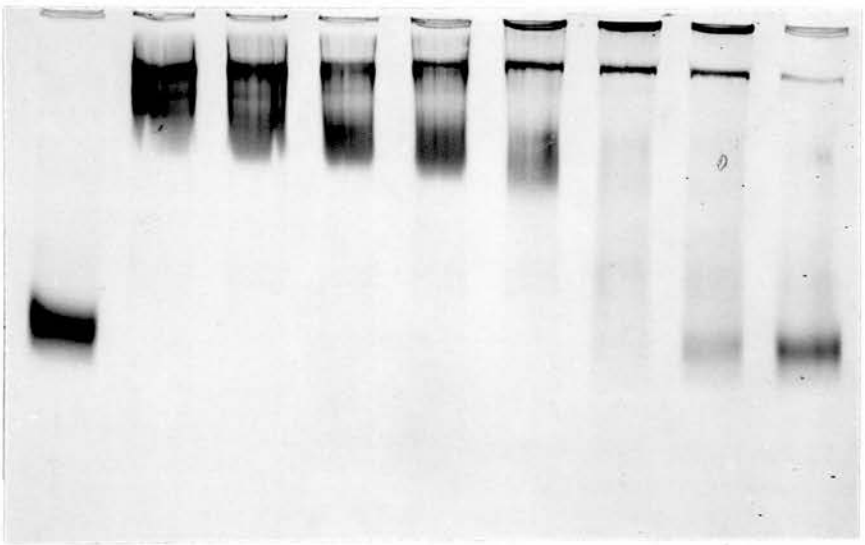
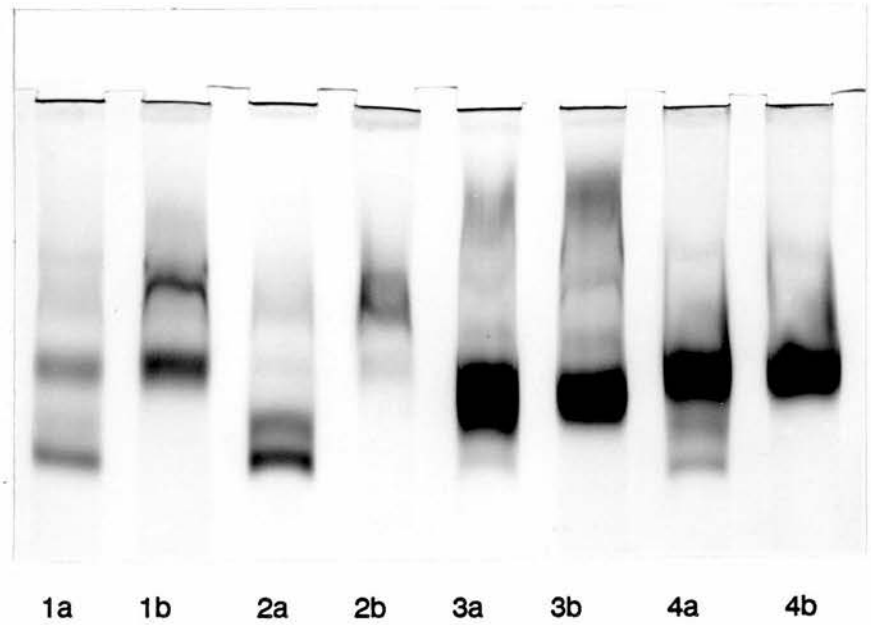


Figure 2.12. Supernatants of samples without antibody (tracks 1a, 2a, 3a and 4a) and samples with antibody to liver/bone ALP (tracks 1b, 2b, 3b and 4b). See text for details.



Results

(i) Optimisation and assessment of method.

The antibody concentrations and incubation periods used for the different stages of the ELISA method were chosen to give readings between 0 and 2.0 absorbance units for the standards (intestinal ALP activities 0 - 50 U/L). The standard curve (absorbance readings against intestinal ALP activities) was linear up to an intestinal ALP activity of 45 U/L. Samples with activities greater than this were diluted before analysis.

Recovery of intestinal ALP added to plasma was 106% (n = 6, added intestinal ALP = 19 U/L) and 104% (n = 6, added intestinal ALP = 33 U/L). The between-batch precision for intestinal ALP measurement was 10.4% (mean intestinal ALP = 19.2 U/L, range 2 - 122 U/L, n = 34). Two samples with different levels of intestinal ALP activity were assayed on different plates; sample 1 had a mean intestinal ALP of 7.0 U/L (SD = 0.62, CV = 8.8%, n = 15); sample 2 had a mean intestinal ALP of 32.9 U/L (SD = 2.87, CV = 8.7%, n = 16).

(ii) Interference.

Intestinal ALP was measured in plasma samples with strong bone (n = 3) or liver (n = 3) bands on electrophoresis (mean total ALP = 871 U/L, SD = 587 U/L, range 250 - 1900 U/L). Mean intestinal ALP was 3.1 U/L, SD = 0.8 U/L, range 2.5 - 4.6 U/L.

(iii) Comparison with other methods.

Figure 2.13 shows intestinal ALP activities measured by ELISA against intestinal ALP measured using L-p-bromotetramisole (BT) for 45 plasma samples. There was greatest discrepancy between ELISA and BT results for samples from some individuals who were either blood group A secretors or blood groups A, B or O non-secretors (closed circles). These samples would be expected to have low levels of intestinal ALP activity (Chapter 5) and on polyacrylamide gel electrophoresis all these samples apart from one showed minimal staining in the intestinal band position. The correlation coefficient between ELISA and BT results with these samples excluded was 0.94 [slope = 1.06 (SE = 0.08); intercept = -10.4 U/L (SE = 2.4)]. Figure 2.14 shows the results for ten samples compared by ELISA and by the immunoprecipitation method. The correlation coefficient was 0.97 [slope = 1.11 (SE = 0.1); intercept = -6.3 U/L (SE = 3.0)].

Figure 2.13. Comparison between the ELISA method and bromotetramisole method (BT) for the measurement of intestinal ALP activity. Open circles represent samples from B, O secretors (for which the correlation line is shown), closed circles are samples from A secretors and A, B, O non-secretors.

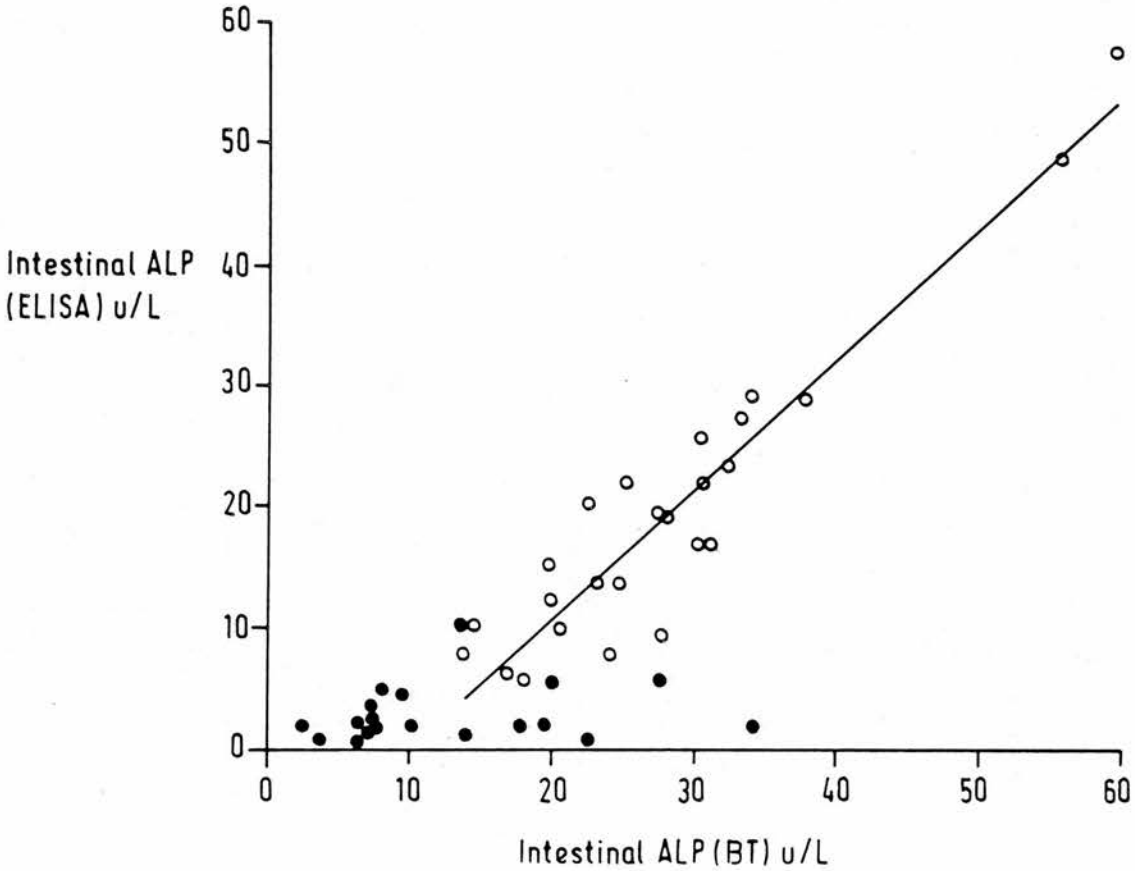
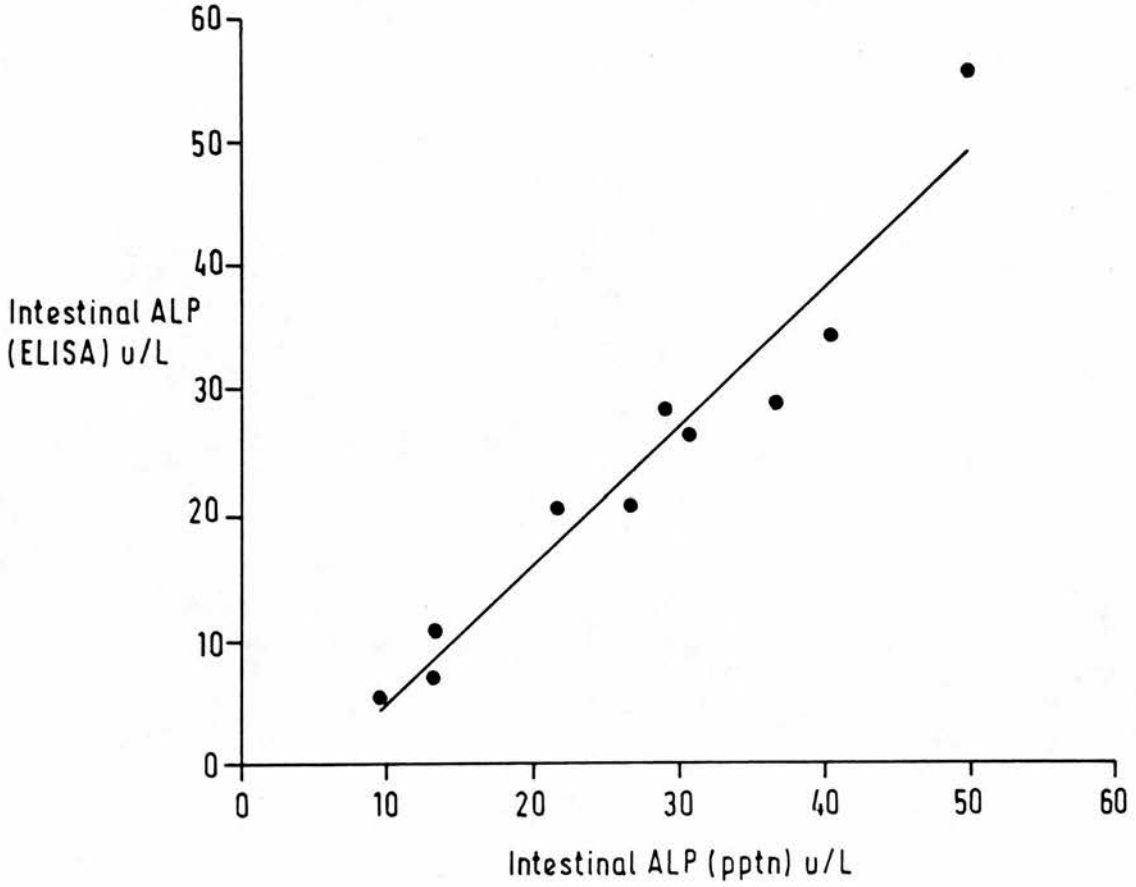


Figure 2.14. Comparison between the ELISA method and the immunoprecipitation method (pptn) for the measurement of intestinal ALP activity.



Discussion

The ELISA method correlated well with the immunoprecipitation method (Figure 2.14) although the results by ELISA tended to be lower, indicating that there may be a slight problem in calibration of the ELISA method. When compared with the BT method there was a close correlation for samples from blood group B, O secretors (Figure 2.13). However, samples from A secretors and A, B, O non-secretors (who generally have low plasma intestinal ALP activities) gave low activities when measured by ELISA but quite high activities when measured by the BT method. The absence of an intestinal band on electrophoresis for the majority of these samples suggests that the ELISA results are correct and that the BT method is giving spuriously high results for these samples. The reason for these findings was not clear.

The immunoprecipitation method was precise but not sensitive as all samples were diluted with S-500-linked immunoglobulin before intestinal ALP measurement. The method was not suitable for measurement of intestinal ALP in large numbers of samples. In addition, it relied on reagents (antibody to liver/bone ALP, S-500-linked immunoglobulin) which are not widely available. Because the antibody to liver/bone ALP does not complex high-molecular-mass ALP (Section 2.6.4), samples with significant amounts of high-molecular-mass ALP will give falsely elevated intestinal ALP activities. In this section, it was used mainly as an independent measure of intestinal ALP for comparison with the ELISA and BT methods.

The bromotetramisole method was quick and easy to perform and suitable for the analysis of large numbers of samples. Bromotetramisole is stable (for at least 1 year when stored in aliquots at -20^{0}C), readily available and inexpensive. The method showed acceptable between-batch precision. However, it was not specific - the presence of placental ALP giving falsely elevated intestinal ALP activities (Section 2.7.1). In addition, as mentioned above, some samples gave spuriously elevated intestinal ALP activities by the BT method.

The ELISA method relies on the use of a monoclonal antibody to intestinal ALP which is not widely available. It also requires the preparation of an intestinal extract for use in calibration. It was found to be less precise than the BT method. Its main advantage over the BT method was its greater specificity - liver, bone and placental ALP (Section 2.7.1) did not interfere with intestinal ALP measurement by ELISA. For this reason, the

ELISA method was chosen for the measurement of intestinal ALP activity in plasma for most of the clinical studies described. In the diabetic study, the BT method was used to measure intestinal ALP since, at that stage, the antibody to intestinal ALP had not been obtained.

2.6. MEASUREMENT OF HIGH-MOLECULAR-MASS ALKALINE PHOSPHATASE

Methods investigated for separating and quantitating high-molecular-mass ALP included gel filtration, ion-exchange chromatography and agarose electrophoresis. An immunoprecipitation method, using antibodies against liver/bone ALP and intestinal ALP, recently described by Maguire and Adnan, 1989, was also evaluated.

2.6.1. Chromatography: FPLC

Introduction

Separation of high-molecular-mass ALP from other ALP forms by using ion-exchange ("Mono Q" column) and a stepwise gradient of LiCl (Schoenau et al, 1986) was described in Section 2.4.3 (Figure 2.1). It was also possible to separate high-molecular-mass ALP by gel filtration (Superose 6 column) on the FPLC system.

Method

The column (Superose 6) was equilibrated at a flow rate of 0.25 ml/min with 50 ml of buffer (20 mmol/L Tris, 50 mmol/L NaCl, pH 8.0). The buffer was filtered and degassed before use. 200 μl of filtered serum were injected on to the column and 0.5 ml fractions collected. ALP activity in the fractions was measured on the Cobas Bio analyser.

Results

High-molecular-mass ALP eluted at 7.1 ml, in the void volume of the column (Blue Dextran 2000, molecular weight 2×10^6 daltons, eluted at 7.5 ml) with the second peak (made up of the other ALP forms) eluting at 15 ml. By using a higher flow rate (0.75 ml/min), it was possible to shorten the run and get separation of high-molecular-mass ALP from other ALP forms in 25 minutes.

A typical chromatogram obtained for a sample from a patient with cholangiocarcinoma is shown in Figure 2.15: peak 1 (high-molecular-mass ALP) eluting between 10 - 14 minutes, elution volume 7.5 - 10 ml; peak 2 (liver ALP) eluting between 20 - 24 minutes, elution volume 15 - 18 ml. Samples with other ALP forms were run under the same conditions; bone, intestinal and placental ALP all eluted between 15 and 18 ml.

2.6.2. Ion-exchange chromatography

Introduction

The two step ion-exchange method described by Crofton and Smith, 1979, was used to measure high-molecular-mass ALP. A brief description of the method is given below.

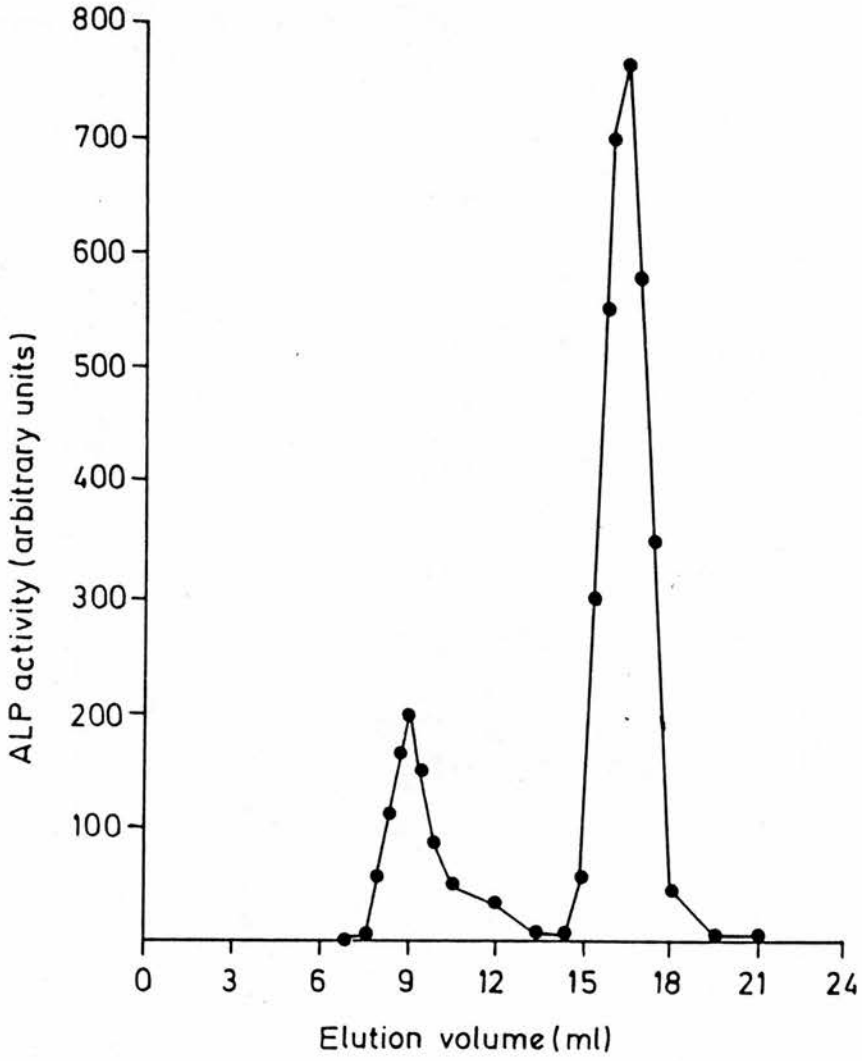
Method

Small columns containing preswollen DEAE-cellulose (bed volume approximately 10 ml) were equilibrated with buffer A (0.1 mol/L NaCl in 0.01 mol/L Tris-HCl buffer, pH 7.5) for 2 hours at a flow rate of 1.4 ml/min. 1 ml of serum, which had been dialysed overnight against buffer A, was added to each column and a 30 ml fraction collected (Fraction I). Buffer B (0.3 mol/L NaCl in 0.1 mol/L Tris-HCl buffer, pH 7.5) was pumped through and a second 30 ml fraction (Fraction II corresponding to the high-molecular-mass form) was collected. ALP activities were measured in Fractions I and II on the Cobas Bio as described for total ALP (Section 2.3) but with an increased sample volume of 80 μl and with 1 mol/L diethanolamine buffer, pH 9.8.

Results

The between-batch precision for high molecular-mass-ALP measurement was 9.3% (SD = 7.4 U/L, range 8 - 292 U/L, n = 10). In 12 samples shown by electrophoresis to contain minimal amounts of high-molecular-mass ALP, but varying proportions of liver, bone and intestinal ALP, high-molecular-mass ALP ranged from 0 - 5.3% of total ALP activity (0 - 6 U/L).

Figure 2.15. Chromatogram of sample containing high-molecular-mass ALP and liver ALP. Separation by gel filtration.



2.6.3. Electrophoresis

Introduction

On polyacrylamide gel electrophoresis, high-molecular-mass ALP remains at the origin (Figure 2.4, track 1a). In this thesis, qualitative polyacrylamide gel electrophoresis was used to determine the presence of high-molecular-mass ALP in plasma. By this method, it was possible to identify samples with high-molecular-mass ALP activities over 5 U/L. High-molecular-mass ALP will enter a 1% agarose gel; agarose gel electrophoresis was investigated to see if it was possible to separate and quantitate high-molecular-mass ALP on this gel system.

Method

A 1% agarose (low electroendosmosis agarose) solution was prepared by heating agarose in Tris-borate buffer until it dissolved. To prevent the gel from falling out during the electrophoretic run, a 7% acrylamide plug was poured first between two glass plates set up in the Gel Casting Unit. The agarose solution was then poured on top of the acrylamide plug, and the well former placed in position. Once the agarose had set, the well former was removed and the sample wells washed with distilled water and then with buffer. Samples were diluted with sucrose and added to the wells as described previously (Section 2.4.5). Bromophenol blue was used as a marker. The gel was run for approximately 3 hours at 200 volts (19 milliamps) in the water-cooled vertical tank using Tris-borate as buffer. Gels were stained and stored as described previously for polyacrylamide gels.

Results

Liver ALP moves ahead of high-molecular-mass ALP on 1% agarose gel (Figure 2.16, track 1a). A scan of this track gave clearly separate peaks which could be quantified. The bone band has a mobility between liver and high-molecular-mass ALP, making it difficult to quantitate high-molecular-mass ALP in a sample which contained bone ALP. Even for samples which contained only liver and high-molecular-mass ALP, the separation was not always as clearly defined as that in track 1a. Sometimes the high-molecular-mass band was diffuse and appeared as a shoulder on the liver peak.

2.6.4. Immunoprecipitation Method

Introduction

At least a proportion of monoclonal antibodies raised to liver ALP cross-react with bone ALP but not with high-molecular-mass ALP (Bailyes et al, 1987). When monoclonal antibodies to liver/bone ALP and to intestinal ALP are added to plasma samples containing the main forms of ALP and the formed complexes removed by solid phased anti-mouse immunoglobulin, high-molecular-mass ALP remains in the supernatant and can be measured.

Method

Plasma samples were set up as follows:

(a) 100 μl of plasma (without antibodies).

(b) 100 μl of plasma (with antibodies; 1:40 antibody to liver/bone ALP, 1:400 antibody to intestinal ALP).

Sephacryl S-500-linked immunoglobulin (100μl) at a dilution of 1:2 was added to (a) and (b). Samples were left for 1 hour at room temperature, mixing occasionally over this period and then centrifuged (3000 xg). Alkaline phosphatase activities of the supernatants were measured. High-molecular-mass ALP activity was calculated from [activity of supernatant of (b)] / [activity of supernatant of (a)] x total ALP. All samples were set up in duplicate.

Results

(i) Optimisation of dilution of antibodies and of sephacryl S-500-linked sheep anti-mouse immunoglobulin.

Final dilutions of monoclonal antibody to liver/bone and to intestinal ALP of 1:40 and 1:400 respectively had to be used to ensure that all the complexed liver, bone and intestinal ALP was removed by the S-500-linked immunoglobulin. As shown earlier, a 1:400 dilution of monoclonal antibody to intestinal ALP complexed all the intestinal band (Figure 2.6, track 5); a dilution of 1:40 of monoclonal antibody to liver/bone ALP complexed all the bone band (Figure 2.11, track 6). Table 2.5 gives supernatant ALP activities for the different dilutions of S-500-linked immunoglobulin. The activities were similar for the 1:2 and 1:4 dilution of S-500-linked immunoglobulin, with activities increasing at higher dilutions of the solid phase.

Table 2.5. Supernatant ALP activity at different dilutions of S-500-linked immunoglobulin.

Dilution of S-500-linked immunoglobulin	Supernatant ALP activity (U/L)
1 : 2	44
1 : 4	46
1 : 8	72
1 : 16	128
1 : 32	200
1 : 64	260

(ii) Agarose gel electrophoresis.

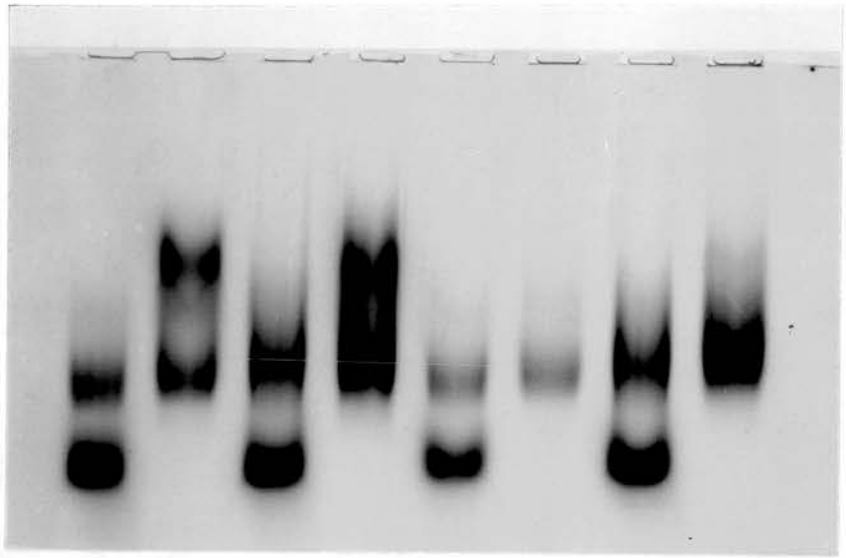
Two plasma samples with liver and high-molecular-mass ALP (samples 1 and 2) were set up without and with antibody to liver/bone ALP (1:40). Samples 1 and 2 were also set up with S-500-linked immunoglobulin (at a dilution of 1:2) as described above. The samples and supernatants were run on agarose gel electrophoresis.

Figure 2.16 shows the separation of liver and high-molecular-mass ALP in the two plasma samples. For both samples, the liver band was retarded by the monoclonal antibody to liver/bone ALP while the mobility of the high-molecular-mass band was unaffected (compare track 1a with track 1b and track 2a with track 2b). When S-500-linked immunoglobulin was added to the sample with antibody, only the high-molecular-mass band remained in the supernatant (compare track 1c with 1d and track 2c with 2d).

(iv) Interferences.

In 12 plasma samples, shown by electrophoresis to contain minimal amounts of high-molecular-mass ALP, but varying proportions of liver, bone and intestinal ALP, high-molecular-mass ALP ranged from 0 - 3.7% of total ALP (0 - 4 U/L).

Figure 2.16. Agarose gel electrophoresis of samples 1 and 2.
Tracks 1a and 2a: unmodified samples; tracks 1b and 2b: samples with antibody to liver/bone ALP; tracks 1c and 2c: supernatants of samples with S-500-linked immunoglobulin; tracks 1d and 2d: supernatants of samples with antibody and S-500-linked immunoglobulin.



(v) Comparison with ion-exchange method.

Figure 2.17 shows the results obtained for high-molecular-mass ALP by the immunoprecipitation method and by ion-exchange [slope = 0.96 (SE = 0.04); intercept = -1.04 U/L (SE = 4.5); r = 0.93; n = 78]. The samples were from patients with obstructive liver disease. The results agreed in most samples. However, three samples had results by the immunoprecipitation method which were approximately half those obtained by ion-exchange.

(vi) Precision.

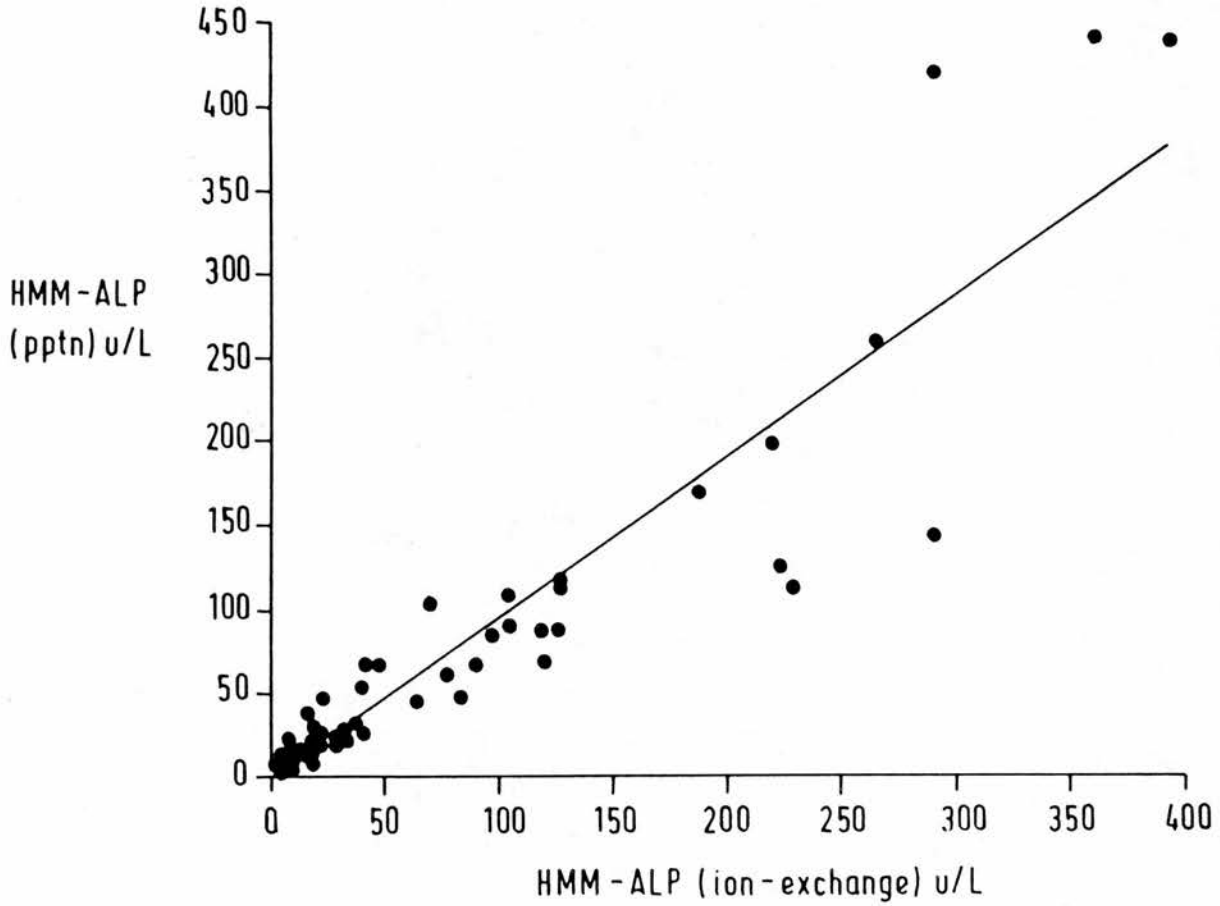
Between-batch precision for the immunoprecipitation method was 12.5% (mean high-molecular-mass ALP = 42 U/L, range 3 - 142 U/L, n = 16).

Discussion

The immunoprecipitation method gave values for high-molecular-mass ALP which compared well with the ion-exchange method for most samples (Figure 2.17). However, there were cases where the high-molecular-mass ALP activity was significantly lower when measured by the immunoprecipitation method compared to ion-exchange. In addition, some samples gave higher values by the immuno-precipitation method. Placental ALP, if present, would be expected to give a positive interference in the immunoprecipitation method. None of these samples contained placental ALP (ALP activity remaining after heating the plasma samples for 10 minutes at 65^{o}C <1 U/L for all samples). High-molecular-mass ALP is known to be heterogeneous (De Broe et al, 1985) and the forms released in obstructive liver disease may vary depending on the cause of obstruction. This may explain the differences in results obtained for the two methods since the methods are based on entirely different principles.

Although gel filtration gave excellent separation between high-molecular-mass ALP and other ALP forms, the repeated injections of serum samples on to the column resulted in increased back-pressures. To reduce this, the column had to be cleaned regularly (with 70% formic acid) and the filter at the top of the column had to be changed. Because of its unsuitability for serum samples, the FPLC method was not investigated further.

Figure 2.17. Comparison between the immunoprecipitation method (pptn) and ion-exchange method for the measurement of high-molecular-mass (HMM) ALP.



The ion-exchange method was more precise than the immunoprecipitation method although it was labour-intensive and required a larger sample volume. Because of its better precision, the ion-exchange method was chosen for quantitative measurements of high-molecular-mass ALP in the clinical studies for those samples which showed clear staining at the origin on polyacrylamide gel electrophoresis.

2.7. OTHER ALKALINE PHOSPHATASE FORMS

2.7.1. Placental alkaline phosphatase

A plasma sample from a patient in the third trimester of pregnancy was investigated. Figure 2.18 shows the position of the placental band on polyacrylamide gel electrophoresis (track a). However, it is important to note that the position of placental ALP on the gel will vary with the allelic form. Placental ALP was unaffected by the monoclonal antibody to intestinal ALP (track b) but was complexed by the antibody to placental ALP (track e). Tracks c and d show the effect of neuraminidase treatment alone and with added antibody to intestinal ALP respectively. The patterns in both tracks are the same; the placental band is retarded and moves in the same position as the liver band. Thus, quantitation of liver and bone ALP in the presence of placental ALP is possible if an antibody to placental ALP is added prior to neuraminidase treatment.

Total ALP and "BT-inhibited ALP" were measured in plasma samples (n = 4) containing placental ALP (mean activity remaining after heating for 10 minutes at 65°C = 77 U/L, SD = 16). Mean total ALP was 179 U/L and mean "BT-inhibited ALP" was 109 U/L. The same samples gave mean intestinal ALP by ELISA of 5 U/L. Thus, placental ALP interferes in the measurement of intestinal ALP by the BT method but not by ELISA.

"High-molecular-mass ALP" was measured by the immunoprecipitation method in two samples with placental ALP. Mean "high-molecular-mass ALP" in these samples was 109 U/L; it was possible to overcome the interference by placental ALP in the immunoprecipitation method by using an antibody to placental ALP.

2.7.2. Alkaline phosphatase-immunoglobulin complexes

Two plasma samples (samples 1 and 2) which showed slow bands (due to ALP-immunoglobulin complexes) on polyacrylamide gel electrophoresis were investigated to see if the presence of these bands interfered in the measurement of the main ALP forms. Figure 2.19 shows that in sample 1, ALP activity appeared as a slow band with the rest of the activity in the liver and bone bands (track 1a); while in sample 2 the majority of the ALP activity was present as a slow band (track 2a). Addition of antibody to liver/bone ALP to the two samples complexed the slow band and the liver and bone bands (tracks 1b and 2b) while the antibody to intestinal ALP had no effect on the slow band in either sample (tracks 1c and 2c). Neuraminidase treatment (15 minutes at 37°C) resulted in clear separation of liver and bone bands in sample 1, but for both samples had a minimal effect on the slow band (tracks 1d and 2d).

The slow bands did not interfere in the measurement of intestinal ALP by the ELISA method (intestinal ALP in sample 1 = 3.3 U/L, in sample 2 = 2.6 U/L). "High-molecular-mass" ALP measured by the immunoprecipitation method in samples 1 and 2 was 18 U/L (9.8% of total ALP) and 36 U/L (14.5% of total ALP) respectively. Therefore in sample 2, where all the ALP activity was present as a slow band, the immunoprecipitation method gave a value for high-molecular-mass ALP of only 14.5% of total ALP. This result suggests that the presence of a slow band in plasma does not interfere with the immunoprecipitation method. Crofton and Smith, 1979, showed that ALP-immunoglobulin complexes do not interfere in the ion-exchange method.

In conclusion, it was possible to recognise the presence of placental ALP and ALP-immunoglobulin complexes by polyacrylamide gel electrophoresis. Some cancer-associated forms will also be detected e.g. fetal-intestinal (Kasahara) isoenzyme by its fast mobility. Others (PLAP and PLAP-like forms) can be identified by their heat stability. However, it was not the purpose of the present study to identify or measure these forms in plasma.

Figure 2.18. Sample with placental ALP and small amounts of liver and bone ALP. Track a: unmodified sample; track b: sample with monoclonal antibody to intestinal ALP; track c: neuraminidase-treated sample; track d: sample with antibody to intestinal ALP added, prior to neuraminidase-treatment; track e: sample with antibody to placental ALP.

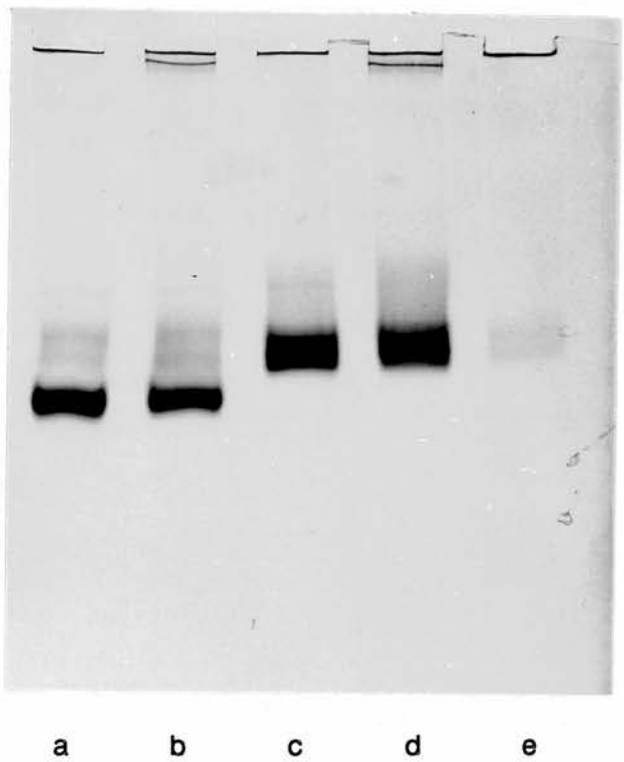
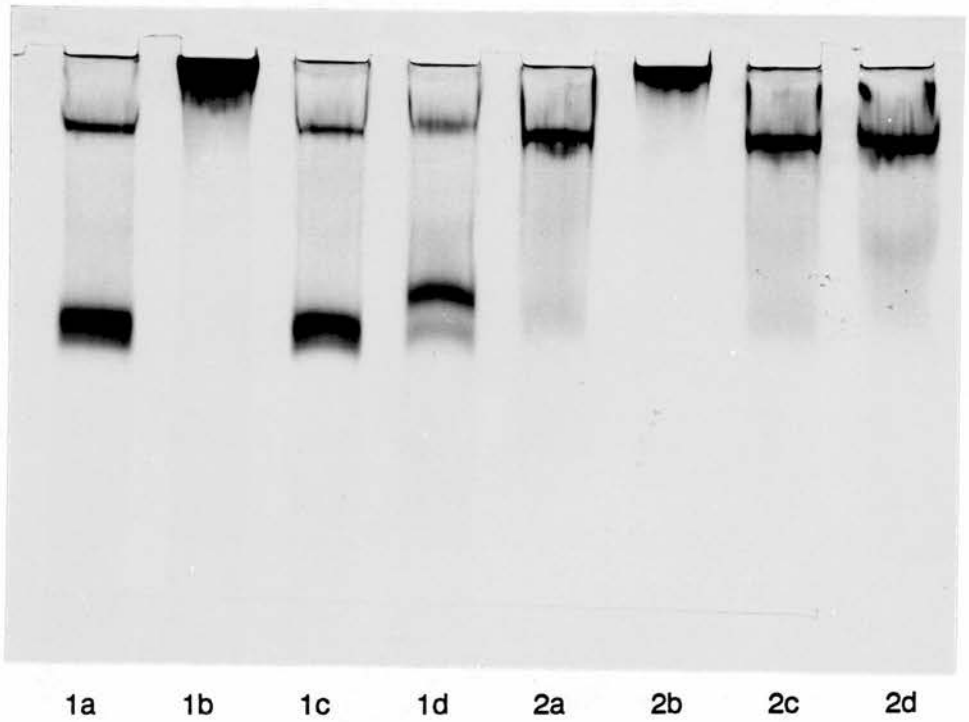


Figure 2.19. Samples 1 and 2 with ALP-immunoglobulin complexes.
Track a: unmodified sample; track b: sample with antibody to liver/bone ALP; track c: sample with antibody to intestinal ALP; track d: neuraminidase-treated sample.



CHAPTER 3

RADIOIMMUNOASSAY FOR OSTEOCALCIN

This chapter describes the development and assessment of a radioimmunoassay for the measurement of plasma osteocalcin. Commercial kits for the measurement of osteocalcin are available but these are expensive, with an average cost of £10 per sample. An in-house radioimmunoassay, once developed, will be inexpensive in terms of reagent costs, thus making it possible to carry out large numbers of osteocalcin measurements.

3.1. MATERIALS

The materials have been divided up into those used for the purification of osteocalcin, the preparation of the antisera and the radioimmunoassay.

Purification of osteocalcin

Gel filtration; G-100 Sephadex (stock no. G-100-120): Sigma.
Ion-exchange; DEAE Sephadex (stock no. A-25-120): Sigma.
SDS-polyacrylamide gel; acrylamide, NN'-methylene-bisacrylamide, ammonium persulphate, TEMED, sodium dodecyl sulphate (SDS): BDH. Coomassie Brilliant Blue R (no. B-0630): Sigma.
SDS Molecular Weight Markers: MW-SDS-17 Kit: Sigma.
Protein determination; Coomassie Brilliant Blue G-250

Preparation of antisera

New Zealand white rabbits: Charles River Breeding Laboratories, UK.
Polyvinylpyrrolidone (stock no. PVP-40), Freund's adjuvant, complete (no. F-4258) and incomplete (no. F-5506): Sigma.

Radioimmunoassay

[^{125}I]-labelled sodium iodide: Amersham International, Bucks., UK.

Sephadex G-25: Sigma

Normal rabbit serum (product no. S119-205) and donkey anti-rabbit IgG (product no. S022-220): SAPU.

Miscellaneous

All other reagents not mentioned above were ordered from Sigma or BDH. All buffers were made up of AnalaR grade reagents from BDH.

3.2. EQUIPMENT

Peristaltic pump and fraction collector: LKB.

Freeze dryer: Edwards High Vacuum.

Centrifugal analyser: Cobas Fara.

Flame photometer: Instrumentation Laboratories.

Vertical electrophoresis tank: LKB.

Gamma counter: LKB (Multigamma counter).

3.3. STANDARD METHODS

3.3.1. Measurement of protein concentration

The protein concentrations of the extract and column fractions (Section 3.4.1) were measured using the Bradford dye binding assay (Bradford, 1976) adapted for use on the Cobas Fara. Coomassie Brilliant Blue G-250 (prepared as described by Bradford) was filtered before use. Absorbance readings were taken at 595 nm after a 100 second incubation at 37^{o}C of sample with reagent. Bovine serum albumin was used to prepare a standard curve over the range 0 to 100 mg/L.

3.3.2. Gel filtration chromatography

Sephadex G-100 was left to swell in 50 mmol/L ammonium bicarbonate and, as a 50% slurry, was poured into a 4.4 x 100 cm glass column. The Sephadex G-100 column was equilibrated for 60 hours at 4°C with 50 mmol/L ammonium bicarbonate, pH 6.0 at a flow rate of 30 ml/hour.

3.3.3. Ion-exchange chromatography

DEAE Sephadex and Sephadex G-25 were left to swell overnight in 100 mmol/L Tris-HCl, pH 7.6. A slurry of DEAE Sephadex was poured on top of a Sephadex G-25 plug in a 2.3 x 50 cm glass column. The column was equilibrated at 4°C for 24 hours with 100 mmol/L Tris-HCl, pH 7.6 at a flow rate of 30 ml/hour.

3.3.4. SDS-urea acrylamide gel electrophoresis

SDS-urea acrylamide gels [12.5% acrylamide (1:10 bisacrylamide to acrylamide), 0.1% SDS, 8 mol/L urea] were prepared and electrophoresis carried out as described in the Sigma Technical Bulletin No. MWS-877P (method of Swank and Munkres, 1971). The buffer contained 0.1% SDS and 0.1 mol/L phosphoric acid adjusted to pH 6.8 with 1 mol/L Tris. The protein concentration of samples was adjusted to 1 g/L before running the samples on the gel. Gels were stained using Coomassie Brilliant Blue R as described in the Technical Bulletin.

Molecular weight markers were included on each gel (MW-SDS-17 Molecular Weight Marker Kit: Sigma). The kit was made up of horse heart myoglobin (M_r 16,950 daltons) and its cyanogen bromide cleavage products (Fragment I + II, M_r 14,400 daltons; Fragment I + III, M_r 10,700 daltons; Fragment I, M_r 8,160 daltons; Fragment II, M_r 6,210 daltons). The M_r values are those given by Schagger and von Jagow, 1987, and McFarlan et al, 1989. Fragment III (M_r 2,510 daltons) although present in the peptide mixture is not visualised by the staining procedure (communication from Sigma).

3.4 PURIFICATION OF OSTEOCALCIN

3.4.1. Methods and results

(i) Preparation of bone extract.

Osteocalcin was isolated and purified as described by Price et al, 1976, with some modifications (Poser et al, 1980). A bullock femur was obtained from a local abattoir; the bone was cleaned of connective tissue and marrow and then reduced to a fine powder using an electric saw. The powder (100g) was washed several times with distilled water and then stirred with 500 ml of 20% v/v of formic acid for approximately 3 hours at 4°C. The mixture was then passed through glass wool. The extract was dialysed at 4°C against several changes of 50 mmol/L ammonium bicarbonate (total volume 35 litres) over 3 days until all the formic acid had been removed. The dialysed extract was then centrifuged for 1 hour at 10,000 xg and the clear solution placed on a freeze dryer. The freeze-dried extract was taken up in 30 ml of 50 mmol/L ammonium bicarbonate; the protein dissolved to give a clear solution when the solution was acidified to pH 6.0. The protein concentration of the extract was 2 g/L (total protein content of the extract = 60 mg).

(ii) Chromatography of bone extract.

a. Sephadex G-100.

The extract (30 ml) was pumped onto the G-100 column and 5 ml (10 min) fractions collected. The protein concentration of alternate fractions was measured on the Cobas Fara. Figure 3.1 shows the protein profile obtained for the extract. To monitor the proteins present in the peaks, fractions at several points on the profile were run on an SDS-urea acrylamide gel electrophoresis. Fractions across the second broad peak had a similar appearance and contained the protein bands with the fastest mobility during electrophoresis on SDS-urea acrylamide gel. Osteocalcin migrates ahead of all other proteins present in bone extract on 12.5% acrylamide (Price et al, 1976). The broad peak (fraction numbers 190 to 280) was therefore pooled. The pooled peak had a protein concentration of 45 mg/L (total volume = 450 ml). This was dialysed against two changes of 100 mmol/L Tris-HCl, pH 7.6 (total volume of 20 litres).

b. DEAE Sephadex.

The pooled peak from the Sephadex G-100 column was added to the DEAE Sephadex column and the column was washed with 100 mmol/L Tris-HCl. Protein was not detected in fractions collected during the addition of the pooled peak or the washing of the column. A salt gradient from 0 to 750 mmol/L NaCl in Tris-HCl buffer was used to elute the protein from the column. 5 ml (10 min) fractions were collected until fraction number 130. Sodium concentrations were measured on a flame photometer in every fourth fraction (diluted 1:5). The protein concentration of alternate fractions was measured as described in Section 3.3.1.

Figure 3.2 shows the protein profile for the DEAE Sephadex column from the point where the salt gradient was started. The major peak eluted at a sodium concentration of 480 mmol/L. Fractions across the peak were run on an SDS-urea acrylamide gel. All the fractions showed two bands very close together with no other protein bands present. The peak was pooled (fraction numbers 70 to 100; total volume approximately 150 ml; protein concentration = 120 mg/L).

(iii) SDS-urea acrylamide gel electrophoresis.

Figure 3.3 shows the gel photograph for the bone extract, the Sephadex G-100 peak, the DEAE sephadex peak and molecular weight markers. In all cases, track a was loaded with 20 μl of sample and track b with 40 μl of sample. The bone extract (tracks 1a and 1b) contained several protein bands of molecular weight greater than myoglobin (M_r 16,950 daltons); these bands were not present in the tracks of the Sephadex G-100 peak (tracks 2a and 2b) or in the tracks of the DEAE Sephadex peak (tracks 3a and 3b). The track of the Sephadex G-100 peak had a similar appearance to the track of the DEAE Sephadex peak and showed a strong band with a fainter band behind it. No other protein bands were present. The strong band moves further than myoglobin fragment I (M_r 8,160 daltons). The molecular weight of the protein can be roughly estimated, from the position of the markers, as between 7,000 and 8,000 daltons.

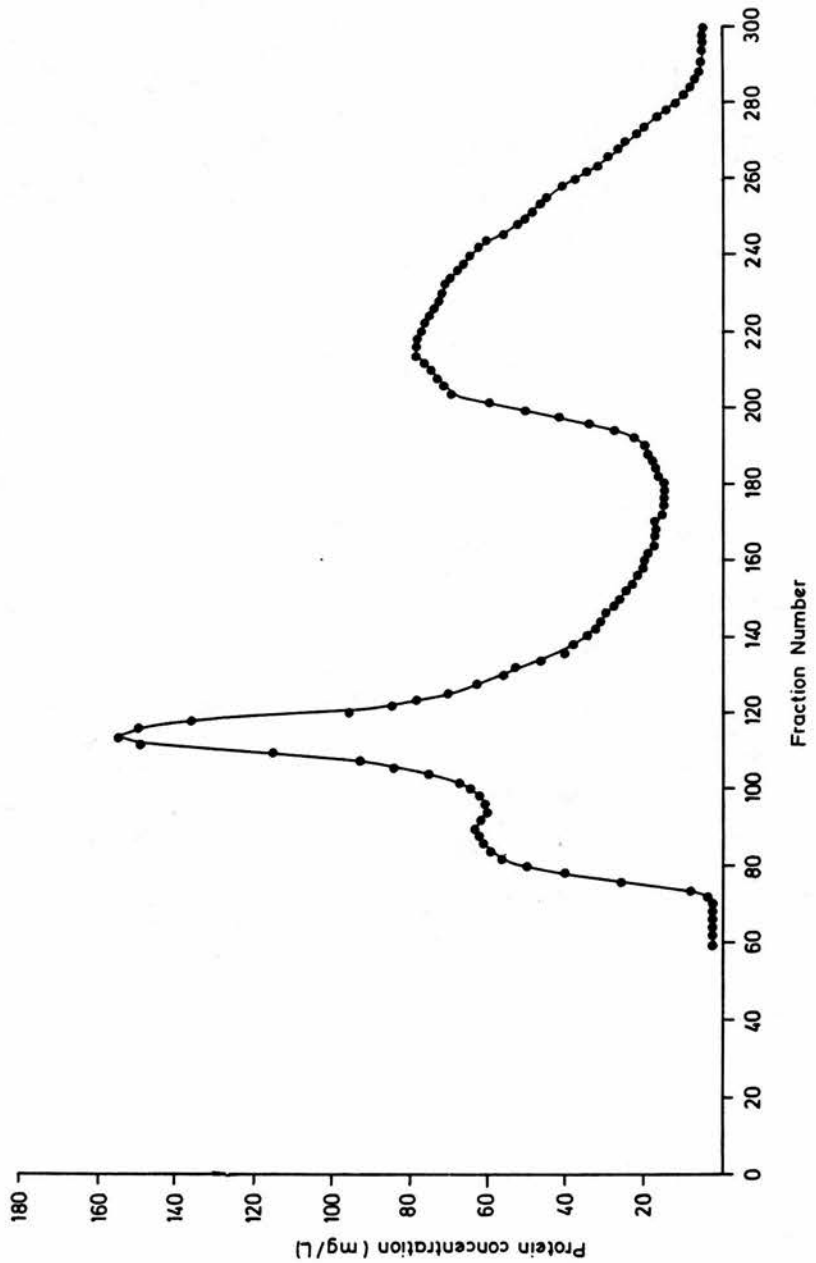


Figure 3.1. Protein profile of bone extract on Sephadex G-100.

Figure 3.2. Protein profile of the pooled peak (from the Sephadex G-100 column) on the DEAE Sephadex column.

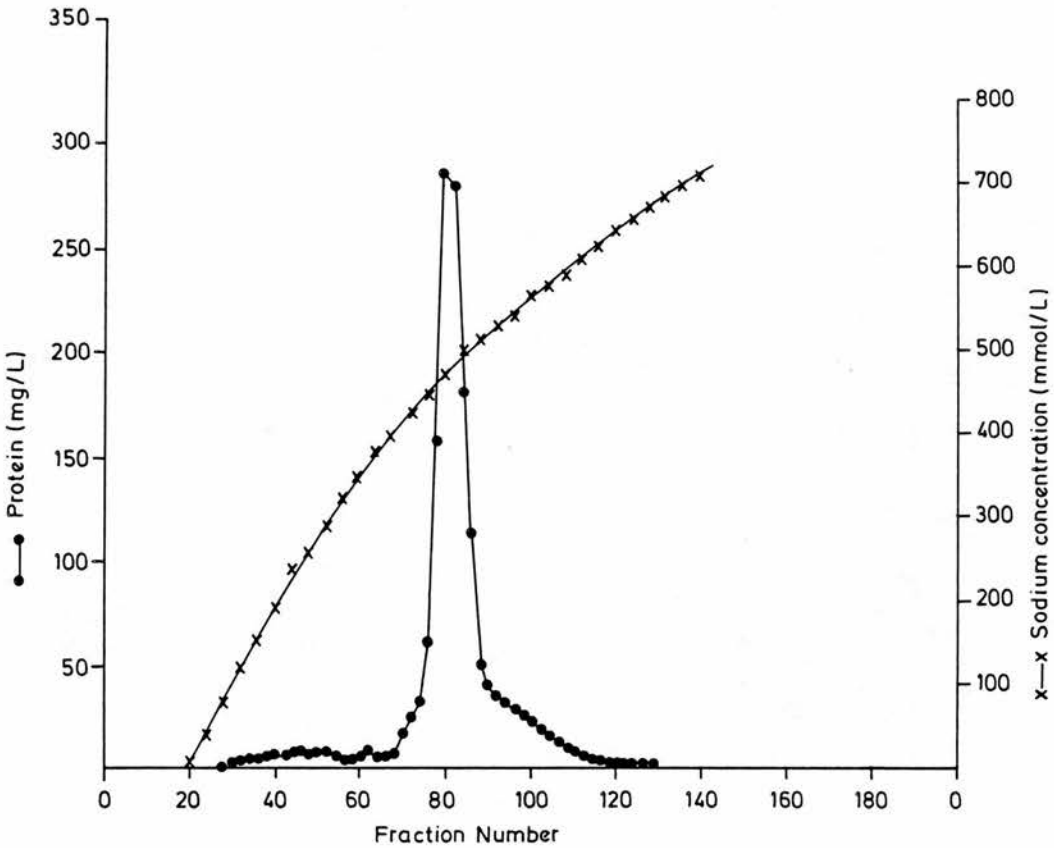
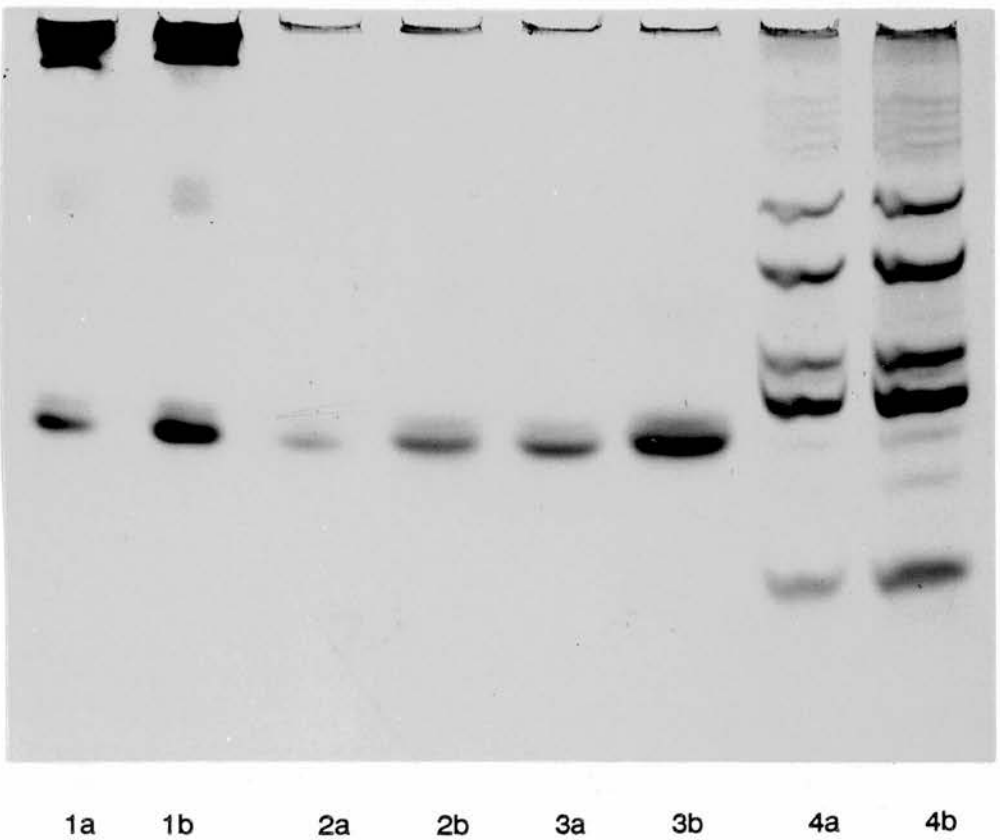


Figure 3.3. SDS-urea acrylamide gel electrophoresis.
Bone extract (tracks 1a,1b); Sephadex G-100 peak (tracks 2a,2b); DEAE Sephadex peak (tracks 3a,3b) and molecular weight markers (tracks 4a,4b). Markers in order of increasing mobility: Myoglobin (M_r 16,950 daltons); Fragment I + II (M_r 14,400 daltons); Fragment I + III (M_r 10,700 daltons); Fragment I (M_r 8,160 daltons); Fragment II (M_r 6,210 daltons).



(iv) Measurement of osteocalcin.

Osteocalcin was measured in an aliquot from the DEAE Sephadex peak using a commercial radioimmunoassay kit (CIS UK Ltd - Section 4.1.6). The aliquot was diluted 1:10,000 with the zero standard of the kit. The assay was carried out as described by the manufacturer, but without using a non-specific binding tube. The concentration of osteocalcin in the pooled DEAE Sephadex peak was 116 mg/L [protein concentration of peak = 120 mg/L - Section 3.4.1.(ii)b].

(v) Storage of osteocalcin.

The DEAE Sephadex peak was dialysed against two changes of 10 mmol/L ammonium acetate, pH 7.0 (total volume = 10 litres). The dialysed peak was placed on the freeze dryer, the lyophilised peak taken up in 30 ml ammonium acetate and stored in vials (5 ml portions) at -70°C. Each vial contained approximately 0.5 g/L protein (2.5 mg protein).

3.4.2 Discussion

Osteocalcin was extracted from bone using formic acid and then purified by gel filtration and ion-exchange chromatography. The majority of interfering proteins were removed in the gel filtration step so that on SDS-urea acrylamide gels only a strong band (with a fainter band behind it) remained (Figure 3.3: tracks 2a and 2b). The DEAE Sephadex step concentrated the protein into a narrow peak which eluted at a sodium concentration of 480 mmol/L (Figure 3.2). The amount of protein present at each stage of the purification was as follows: bone extract - 60 mg, Sephadex G-100 peak - 20 mg, DEAE Sephadex peak - 18 mg of protein.

The majority of the protein in the DEAE Sephadex peak (total protein = 18 mg) was made up of osteocalcin (osteocalcin = 17.4 mg). The yield of osteocalcin from bone (17.4 mg from 100 g of bone powder) was one tenth of that obtained by Price et al, 1976. They dialysed bone against 0.5 M EDTA at 4°C for 8 - 10 days. This may be more efficient in extracting osteocalcin from bone compared to formic acid. However, they used a non-specific method (measurement of gamma carboxyglutamic acid in alkaline hydrolysates) to estimate the amount of osteocalcin present. I found the recovery of osteocalcin from bone to be closer to that found by Power et al, 1989.

They obtained 12.1 mg of purified osteocalcin from 100 g of dried bone powder. They extracted the bone powder with ammonium EDTA for 16 hours and measured osteocalcin concentrations by radioimmunoassay.

Osteocalcin appeared as a double band (strong band ahead of a weak band) on SDS-urea acrylamide gel electrophoresis. Worsfold et al, 1988, also found that purified osteocalcin resolved into a close doublet on SDS-urea acrylamide gels. The strong band (Figure 3.3) migrated ahead of myoglobin fragment I (M_r 8,160 daltons), giving osteocalcin an estimated M_r of 7,000 - 8,000 daltons. This is higher than the true M_r of bovine osteocalcin (5,800 daltons) established by sequence analysis. The SDS-urea acrylamide gel system can determine the molecular weight of peptides within ±18% of the true molecular weight (Swank and Munkres, 1971) and is not suitable for the accurate determination of molecular weight. It was used mainly to follow the purification of osteocalcin and to show that the purified protein was composed of one main band.

3.5. PREPARATION OF ANTISERA

One vial (0.5 g/L protein in 10 mmol/L ammonium acetate) was placed on the freeze dryer overnight. The lyophilised protein was dissolved in 0.5 ml of 0.15 mol/L NaCl, which was acidified to pH 6.0. The protein was adsorbed onto polyvinylpyrrolidone (PVP) to make it more antigenic (Worobec et al, 1972). 2.5 ml of 50% w/v PVP was added to the protein and the solution was placed on a rotary mixer for 2 hours at room temperature. The protein solution was added to 3 ml of Freund's complete adjuvant (kept on ice) and mixed to give an emulsion. Three New Zealand white rabbits were immunized; 1.5 ml (0.6 mg protein) was injected intradermally at multiple sites into each rabbit.

Six and 8 weeks later, the rabbits were given boosting injections of 0.3 mg protein (half the protein used for the first injection). The emulsions were prepared as before but with Freund's incomplete adjuvant. All three rabbits produced antisera to osteocalcin 10 weeks after the first injection. The rabbits were anaesthetized and bled by cardiac puncture. 50 ml of blood was obtained from each rabbit. The separated serum was preserved with 0.02% azide and stored at -70°C.

3.6. PREPARATION OF LABELLED OSTEOCALCIN

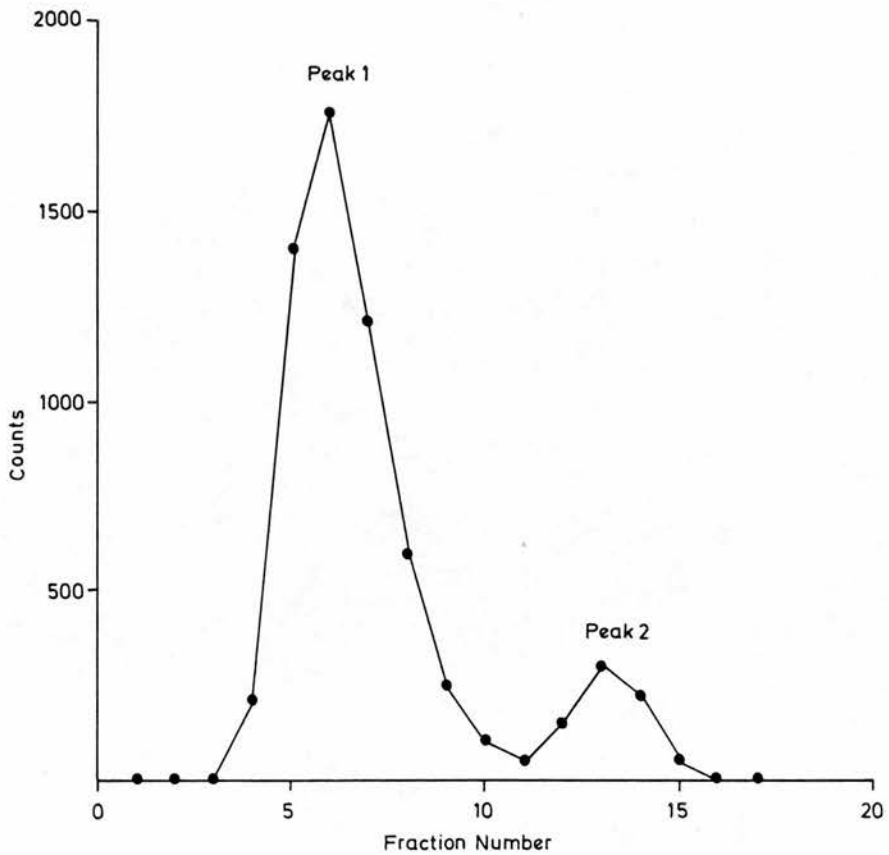
[^{125}I] Osteocalcin was prepared using the chloramine-T method (Hunter and Greenwood, 1962). Purified osteocalcin (10 μl; 5 μg) and 50 mmol/L potassium phosphate buffer, pH 7.5 (10 μl) were added to a glass tube followed by ^{125}I-sodium iodide (5 μl; 18.5 MBq) and chloramine-T (10 μl; 16 μg). After 15 seconds, cysteine (100 μl; 16 μg), potassium iodide (10 μl; 100 μg) and assay diluent (250 μl) were added to stop the reaction. The assay diluent consisted of 25 mmol/L potassium phosphate buffer containing 1 g/L bovine serum albumin and 0.2 g/L sodium azide. The contents of the tube were then transferred to a 1 x 12 cm column of Sephadex G-25, which had been equilibrated with assay diluent (as above but containing 10 g/L bovine serum albumin). The column was eluted with assay diluent (0.5 ml/min) and radioactivity in the fractions monitored on a gamma counter.

Figure 3.4 shows the iodination profile obtained from the gel filtration column. The labelled protein appears in peak 1; the free ^{125}I-sodium iodide appears in peak 2. The incorporation of ^{125}I-sodium iodide into the labelled protein was 89%. The specific activity of the labelled osteocalcin was 3.3 MBq/μg of protein. Fractions 6 and 7 were pooled to give the tracer used in the radioimmunoassay described below.

3.7. PREPARATION OF PRECIPITATED SECOND ANTIBODY

Normal rabbit serum (1.5 ml) was added to 20 ml of anti-rabbit IgG (donkey) and mixed overnight on a rotary mixer at room temperature. After centrifugation (10 min at 1,500 xg) the supernatant was discarded, the precipitate washed twice with 20 ml assay diluent (Section 3.6) and then resuspended in 20 ml of the diluent. The precipitated second antibody was stored at 4^{o}C.

Figure 3.4. Iodination profile from Sephadex G-25 column.



3.8. RADIOIMMUNOASSAY

A description of the method used for the measurement of osteocalcin will be given first followed by an account of the optimisation of reagents, the standardisation of the assay and its performance in terms of precision and comparability to a commercially available radioimmunoassay.

3.8.1. Method

All dilutions were made in assay buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 25 mmol/L Na_4 EDTA, 0.05% Tween-20, 0.02% sodium azide, pH 7.2). Antibody was diluted 1:8,000 in assay buffer. Tracer was diluted so that the total counts in the tracer solution were 2×10^4 cpm. Precipitated second antibody was used at a dilution of 1:2 in assay buffer. Tubes for the radioimmunoassay were labelled and antibody, tracer and standard/sample added as follows:

Tubes 1 and 2 (total counts) contained 500 μl of tracer.
Tubes 3 and 4 (non-specific binding tubes) contained 500 μl of tracer and 500 μl of buffer.
Tubes 5 to 20 (8 standards set up in duplicate) contained 50 μl of standards (see below), 500 μl of tracer and 500 μl of antibody.
Tubes 21 onwards contained the unknown samples set up in duplicate.

The tubes were mixed on a multi-vortex and left for 24 hours at 4°C. Precipitated second antibody (100 μl) was added to all tubes, except 1 and 2, and the tubes placed on a shaker for 1 hour. 2 ml of wash solution (0.05% Brij 35) was added to all tubes, except 1 and 2. The tubes were centrifuged for 30 minutes (4°C, 3000 xg), the supernatant decanted and the precipitate counted on a gamma counter. The results were processed using the LKB 1224-Ria Calc. LM RIA evaluation program.

3.8.2. Optimisation

(i) First antibody

Antibody dilutions (doubling dilutions from 1:250 to 1:128,000) were prepared in assay buffer. Diluted antibody (500 μl) was mixed with tracer (500 μl) and the tubes treated as described above.

Figure 3.5 shows the percentage of total counts plotted against final dilution of antibody for the three antisera (numbers 256, 257 and 258). Usable antisera were produced by all three rabbits. Antiserum 258 had a lower antibody titre compared to antisera 256 and 257. Antiserum 257 was used in all radioimmunoassays described since it had a slightly higher titre than antiserum 256.

The following experiment was carried out to check that plasma osteocalcin displaced [^{125}I] osteocalcin from the antibody. Antibody dilutions were prepared as above; to each set of antibody dilutions 50 μl of zero standard (from the CIS UK kit - Section 4.1.6), 50 μl of "normal osteocalcin" plasma (from a blood donor) or 50 μl of "raised osteocalcin" plasma (from a patient with renal failure) were added. The radio-immunoassay was set up and counted as before.

Figure 3.6 shows the antibody dilution curves for the three samples (zero, "normal" and "raised" osteocalcin). A final dilution of antibody of 1:16,000 gave 53% of total counts bound for the zero sample and showed a reduction in binding for the "normal osteocalcin" sample and an even greater reduction in binding for the "raised osteocalcin" sample. This antibody dilution was selected and was used in all the radioimmunoassays described.

Figure 3.5. Percentage of total counts bound against antibody dilution (log scale).
Antisera numbers: 256 (o——o), 257 (x——x), 258 (●——●).

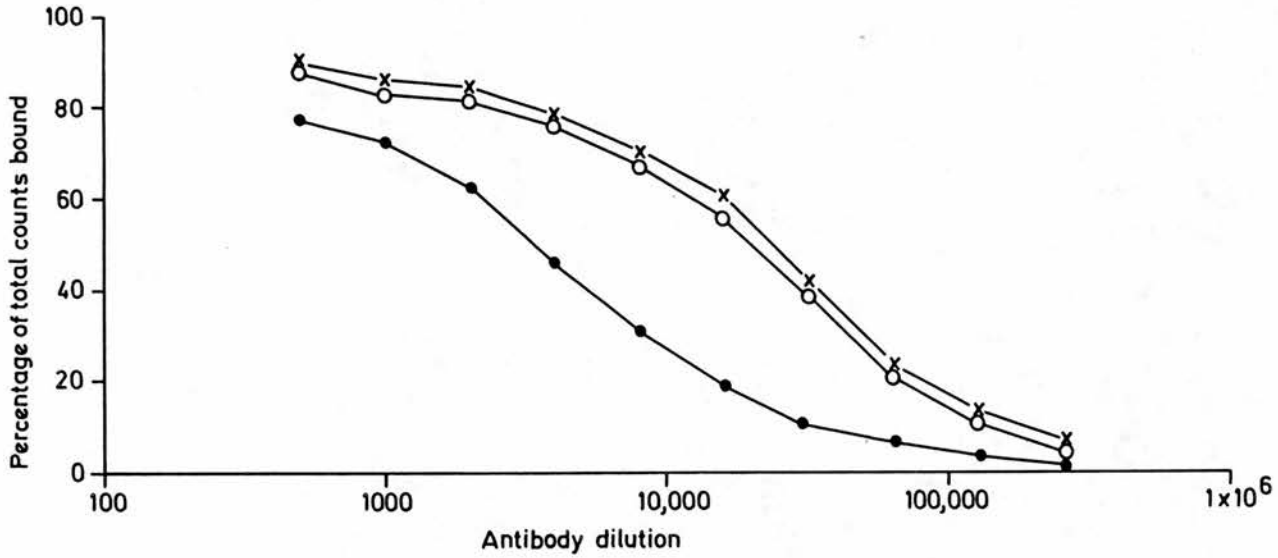
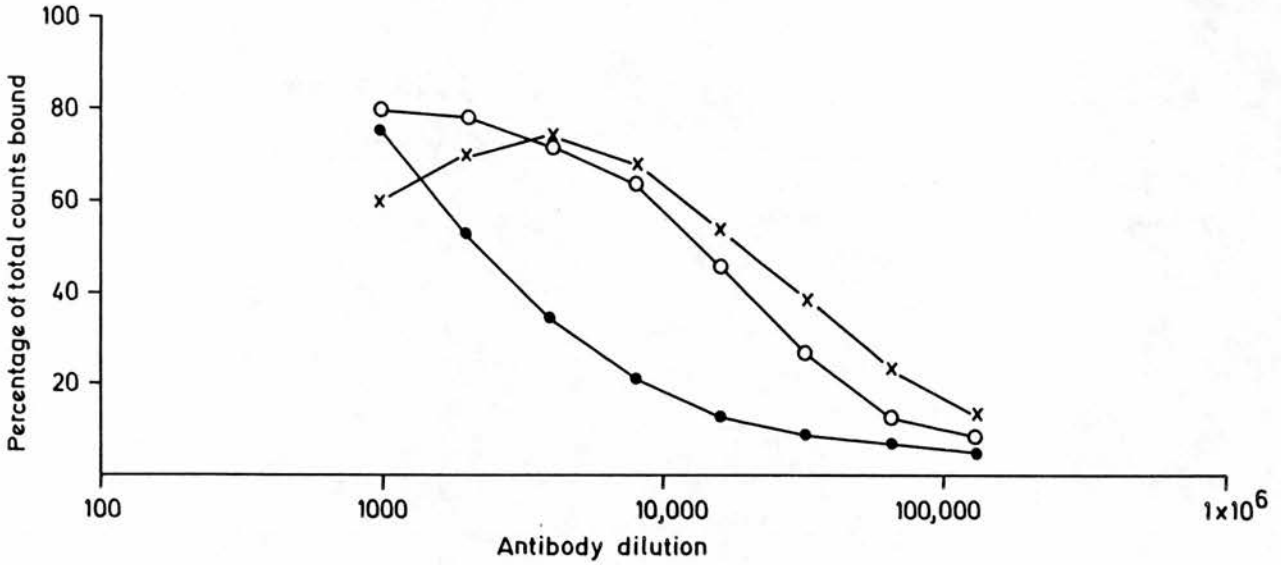


Figure 3.6. Percentage of total counts bound against antibody dilution (log scale).

Samples: 0 standard (x——x), "normal osteocalcin" plasma (o——o), "raised osteocalcin" plasma (●——●).



(ii) Precipitated second antibody.

The radioimmunoassay was set up as described before (using 50 μl of buffer as sample) but, at the separation stage, precipitated second antibody was added as prepared (Section 3.7) and at various dilutions in assay buffer (1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128).

Table 3.1 shows the percentage of total counts bound for undiluted and diluted precipitated second antibody. Although this table shows that it was possible to use the precipitated second antibody at a dilution of 1:8, it was found, in practice, that a dilution of 1:2 had to be used to obtain a firm precipitate. At this precipitated second antibody dilution, it was possible to decant the supernatant without disturbing the pellet.

Table 3.1. Percentage of total counts bound at the dilutions of precipitated second antibody shown.

Dilution of precipitated second antibody	Percentage of total counts bound
Undiluted	52.6
1:2	53.2
1:4	52.5
1:8	51.1
1:16	50.2
1:32	46.9
1:64	32.3
1:128	19.5

3.8.3. Standardisation

The protein concentration of the purified osteocalcin [stored in a vial at -70°C; Section 3.4.1(v)] was determined using the method of Bradford, 1976. The purified osteocalcin was diluted in charcoal-stripped serum to give the following standards: 2.7 μg/L, 5.5 μg/L, 10.9 μg/L, 21.9 μg/L, 43.8 μg/L, 87.5 μg/L, 175 μg/L and 350 μg/L. The charcoal-stripped serum was used as the zero standard.

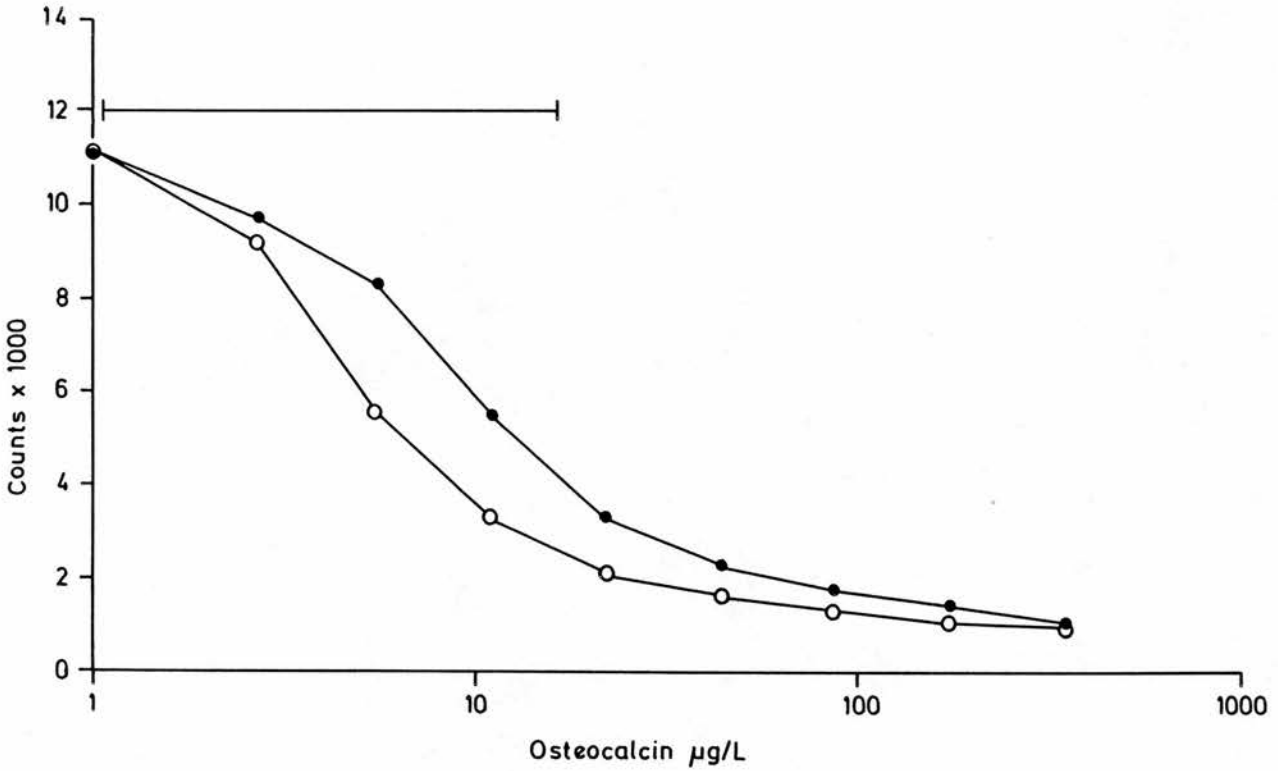
Two runs were set up using the standards:

Run 1 (as before): Antibody (500 μl), tracer (500 μl) and standard (50 μl) were incubated for 24 hours at 4°C. The samples were separated and counted.

Run 2 (delayed tracer addition): Antibody (500 μl) and standard (50 μl) were incubated for 24 hours at 4°C when tracer (500 μl) was added. The samples were incubated for a further 24 hours and then separated and counted.

Figure 3.7 shows the standard curves obtained for Run 1 and Run 2. The "delayed tracer" curve had a steeper slope at a lower concentration of osteocalcin compared to the curve obtained when antibody and tracer were added at the same time. Delayed tracer addition was therefore used when differences between patient groups with low or low/normal levels of osteocalcin were being investigated. The range of osteocalcin concentrations found in blood donors (Chapter 5) is also given.

Figure 3.7. Standard curves for osteocalcin radioimmunoassay, as described in protocol (●——●) and with delayed tracer addition (o——o). Osteocalcin concentration (x axis) is plotted on a log scale. The horizontal line shows the range of osteocalcin concentrations found in blood donors.



3.8.4. Stability of osteocalcin

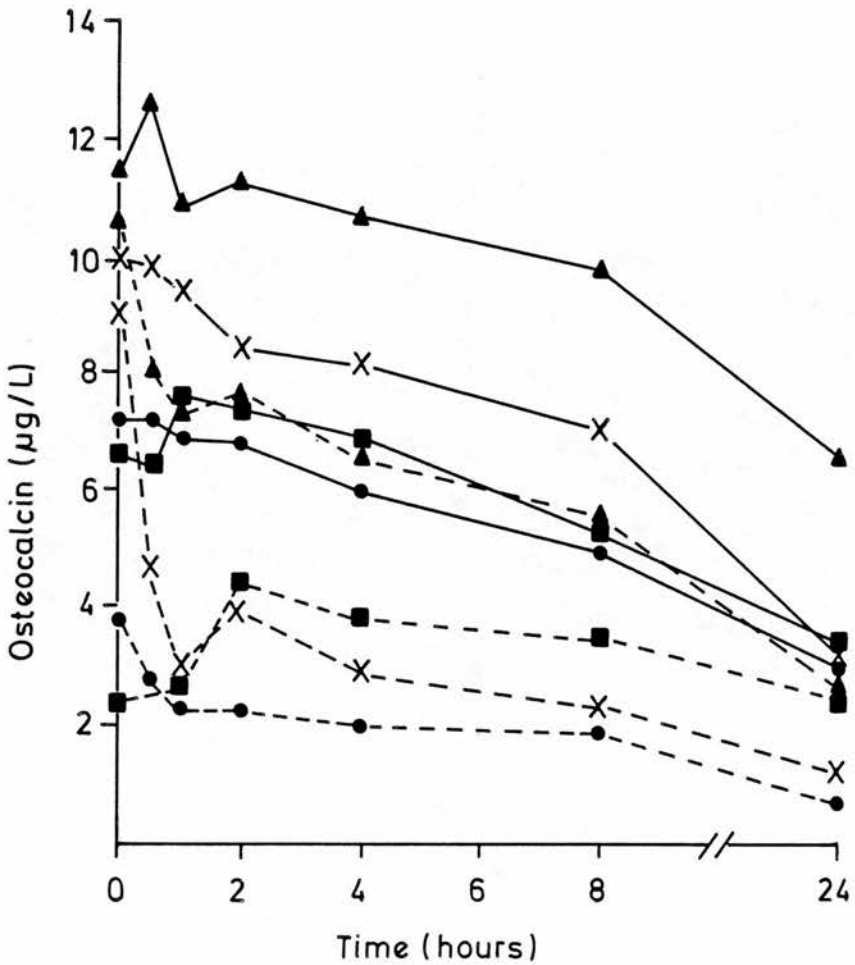
(i) In whole blood (with and without anticoagulant).

Blood was collected from 4 volunteers into lithium heparin tubes and into plain tubes (no anticoagulant). The blood in the plain tubes was left to clot (about 30 min) and both sets of tubes centrifuged (3000 xg for 5 min). An aliquot of plasma and serum were removed from the lithium heparin tube and the plain tube respectively (0 time) and placed at -20°C. Further aliquots were removed (and placed at -20°C) after 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours and 24 hours at room temperature. Osteocalcin was measured in plasma and serum samples using the radioimmunoassay as described above, but with delayed tracer addition.

Figure 3.8 shows the osteocalcin concentrations for the 4 volunteers; plasma and serum was separated from the cells at the times shown. At each time point, osteocalcin levels were lower in the serum sample compared to the plasma sample from the same individual. There was a drop in osteocalcin levels in blood (with or without anticoagulant) with time. This drop was more marked in the plasma sample.

In one of the clinical studies (the liver disease study - Chapter 9) blood was collected from the same patient into lithium heparin tubes and into plain tubes. Plasma and serum were separated at the same time for each patient and stored at -20°C. Osteocalcin was measured in the matched plasma and serum pairs ($n = 20$) and found to be significantly lower ($p < 0.001$; dependent t-test) in the serum samples (mean osteocalcin = 3.8 μg/L, SD = 2.0 μg/L) compared to the plasma samples (mean osteocalcin = 6.4 μg/L, SD = 3.7 μg/L).

Figure 3.8. Stability of osteocalcin in blood collected from 4 volunteers (●, x, ▲, ■). Blood was collected into tubes with (———) or without (- - - -) anticoagulant. Plasma (———) and serum (- - - -) were separated at the times shown and stored at -20°C. Osteocalcin concentrations in plasma and serum samples were measured in one assay.



(ii) In whole blood and in separated plasma.

Blood was collected from a further 4 volunteers into lithium heparin tubes. This was kept either as whole blood or as separated plasma for 30 minutes, 1 hour, 2 hours, 4 hours and 24 hours at room temperature. At 0 time and at the times given above, plasma separated from the whole blood and the previously separated plasma samples were frozen (-20°C). Osteocalcin was measured in these samples. It was also measured in an aliquot of buffer which had been added to a lithium heparin tube to see if lithium heparin interfered in the measurement of osteocalcin.

Table 3.2 shows osteocalcin concentrations expressed as percentages of 0 time values, at each time point for the whole blood sample and the separated plasma sample (the mean of the percentages obtained for the 4 individuals is given in each case). Osteocalcin concentrations in both the whole blood sample and the separated plasma sample decreased with time. This decrease was more marked after 4 hours at room temperature. There was a similar reduction in osteocalcin concentrations in whole blood or in separated plasma. The aliquot of buffer from the lithium heparin tube gave total counts (10,400 cpm) which were greater than those obtained for the zero standard (9,600 cpm).

Table 3.2. Stability of osteocalcin in whole blood and in separated plasma.

Time (hours)	Percentage of 0 time [osteocalcin]*	
	Whole blood	Separated plasma
0.5	99.9	95.2
1	93.6	96.2
2	97.2	94.5
4	91.6	90.2
8	74.5	76.9
24	61.7	55.3

* mean osteocalcin concentration for the four individuals.

3.8.5. Performance of the radioimmunoassay.

Twenty runs were set up for the measurement of osteocalcin concentrations in plasma samples collected from blood donors and from patients for the clinical studies described later. The working range (coefficient of variation < 10% for the measurement of duplicates) for the runs was 2.8 - 65 μg/L. The non-specific binding tube in these runs gave counts which were 3.4% or less of total counts.

Within-batch precision of the radioimmunoassay was checked by carrying out 20 measurements of the same plasma sample in 1 run. The coefficient of variation was 4.5% (mean osteocalcin = 18.5 μg/L, SD = 0.83 μg/L). There was no significant difference (independent t-test; p = 0.2) between the first set of 10 measurements (mean osteocalcin = 18.7 μg/L, SD = 0.82 μg/L) and the second set of 10 measurements (mean osteocalcin = 18.3 μg/L, SD = 0.80 μg/L), indicating that the drift within a run was negligible.

Two plasma samples with "low" and "high" osteocalcin concentrations were divided into aliquots and stored at -20°C. Osteocalcin was measured in these plasma samples in different runs. The "low" osteocalcin plasma gave a CV of 21% (mean osteocalcin = 2.7 μg/L, SD = 0.6 μg/L, n = 15) and the "high" osteocalcin plasma gave a CV of 9.2% (mean osteocalcin = 23.5 μg/L, SD = 2.2 μg/L, n = 14). In aliquots of the pooled plasma sample with a "high" osteocalcin concentration (23.5 μg/L), stored at -20°C, the osteocalcin concentration did not change in ten measurements made over a period of 55 days.

Osteocalcin was measured in different plasma samples in different runs. Between-batch CV for these measurements divided into low, middle and upper ranges is given in Table 3.3. Plasma samples with raised osteocalcin levels were diluted with the zero standard (1:2, 1:4, 1:8 and 1:16) and osteocalcin measured in the undiluted and diluted sample. Table 3.4 shows the results obtained.

Table 3.3. Between-batch precision of osteocalcin measurements.

Range (μg/L)	n	Mean (μg/L)	SD (μg/L)	CV (%)
0.5 - 14	29	7.3	0.9	12.1
14 - 30	13	21.9	1.2	5.6
30 - 65	8	43.8	2.7	6.2

Table 3.4. Linearity of osteocalcin measurements.

Sample	Osteocalcin (μg/L)				
	Undiluted	Dilution*			
		1:2	1:4	1:8	1:16
1	47.2	45.0	52.4	46.4	46.4
2	44.9	63.8	64.4	59.2	51.2
3	29.1	29.6	24.4	19.2	17.6
4	13.8	16.6	18.0	14.4	14.4

* result multiplied by dilution factor.

3.8.6. Comparison with a commercial kit method.

Plasma osteocalcin concentrations were measured in 74 patients with chronic renal failure using a commercial radioimmunoassay (OSTK-PR: CIS UK Ltd, High Wycombe, Buck.) and by the in-house radioimmunoassay described above. The plasma samples had been stored at -20°C and were analysed by the commercial kit method 4 months after collection and by the in-house radioimmunoassay approximately 8 months after collection.

Osteocalcin measurements using the commercial kit were carried out according to the manufacturer's instructions except that a non-specific binding (NSB) tube for each sample was not set up. This resulted in a saving in reagents and thus reduced the cost of each measurement. Osteocalcin levels measured in 12 samples where an NSB tube was included were not significantly different to those obtained without an NSB tube ($p = 0.29$; dependent t-test). Samples with osteocalcin concentrations more than 55 μg/L were diluted in zero standard and osteocalcin measured in the diluted sample.

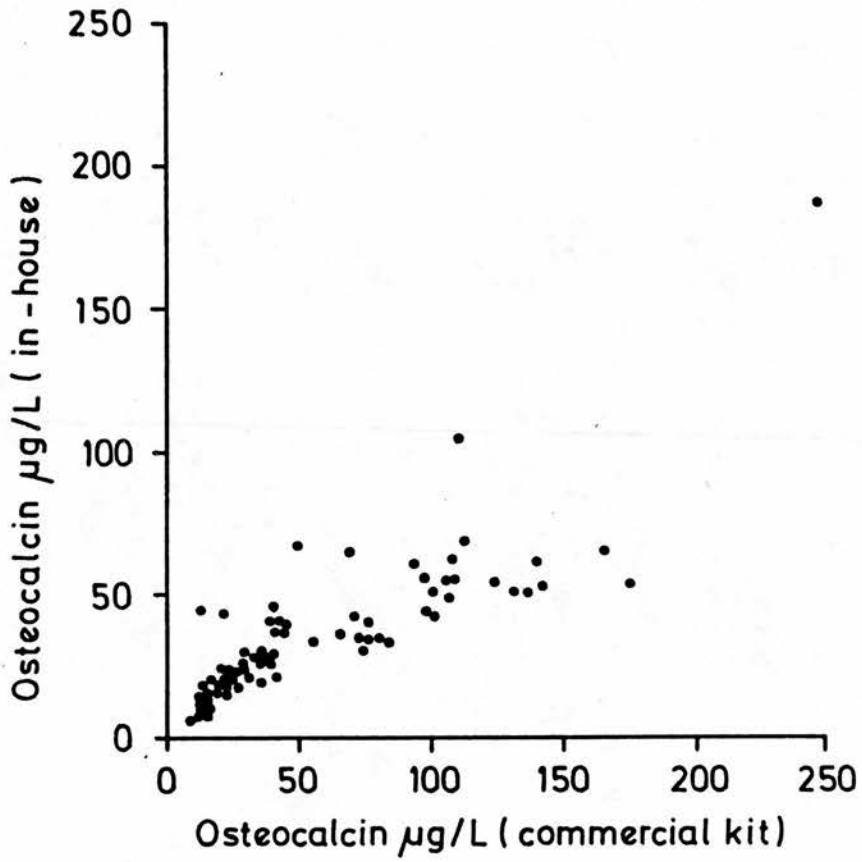
Figure 3.9 shows the comparison between osteocalcin concentrations measured by the in-house radioimmunoassay and osteocalcin concentrations measured by the commercial kit method. The correlation coefficient was 0.83 (slope = 0.44, SE = 0.04; intercept = 9.7, SE = 2.7 μg/L).

3.8.7. Discussion

The radioimmunoassays described above were set up using antibody 257 which was raised against bovine osteocalcin. Tracer (^{125}I-labelled osteocalcin) and the standards were prepared using purified bovine osteocalcin. The antisera cross-reacted with human osteocalcin and were used to measure osteocalcin in the plasma of patients. The C-terminal region of human osteocalcin shows complete homology with bovine osteocalcin; this suggests that the antisera produced, like antisera described by others (Price and Nishimoto, 1980; Gundberg et al, 1985; Power et al, 1989), react with the C-terminal portion of the osteocalcin molecule.

A plasma/serum difference was identified, with consistently lower levels in serum (Figure 3.8). The reason for this is unclear but may well be due to an alteration in the shape of the osteocalcin molecule in serum, resulting in a lower affinity for the antibody. Price and Nishimoto, 1980, Worsfold et al, 1988, and Power et al, 1989, measured osteocalcin by radioimmunoassays using different antibodies in each case but all raised against bovine osteocalcin. They all found no difference in osteocalcin concentrations in heparinised plasma and serum samples taken at the same time. Johansen et al, 1987, found that osteocalcin levels in plasma were 10 - 15% higher than in serum. On the other hand, Egmose et al, 1989, using an enzyme-linked immunosorbent assay for osteocalcin found significantly higher levels in serum.

Figure 3.9. Comparison of in-house radioimmunoassay with a commercial kit for the measurement of plasma osteocalcin.



Osteocalcin levels decreased with time in whole blood collected with or without anticoagulant left at room temperature (Figure 3.8). Osteocalcin levels in separated plasma also decreased with time (Table 3.2). Price and Nishimoto, 1980, showed no change in plasma osteocalcin levels after 24 hours at 25°C, although serum osteocalcin levels fell 19% after 8 hours at 25°C. Reports of other radioimmunoassays in the literature do not give details of stability of osteocalcin in whole blood or in separated plasma and serum. The kit insert from the commercial radioimmunoassay used (CIS UK Ltd) recommends that serum or plasma osteocalcin measurements are performed within 2 hours of collection and, if this is not possible, that the samples are separated and the plasma or serum stored at -20°C until the assay is carried out.

In summary, for the measurement of osteocalcin by the radioimmunoassay described here, plasma should be used. The plasma should be separated and kept at -20°C preferably within 2 hours, but not more than 4 hours after the collection of the blood.

The between-batch precision for the measurement of low osteocalcin concentrations (< 3 μg/L) was poor (CV of 21%). The use of delayed tracer addition would be expected to improve the precision and should be used for the measurement of low levels of osteocalcin. At normal and high osteocalcin levels the between-batch precision was satisfactory and similar to that quoted by Gundberg et al, 1985, (run to run CV = 8.2%) and Worsfold et al, 1988, (interassay CV of mid-range sera = 14%). Samples with high osteocalcin concentrations showed a linear relationship on dilution in most cases (Table 3.4) although sometimes the diluted sample gave a higher value for osteocalcin than the undiluted sample.

The correlation between the in-house radioimmunoassay and the commercial kit method showed a large scatter in the results (Figure 3.9). The kit method gave results which were higher than the in-house method for several samples. The following factors could explain the differences found:

(i) The same samples were measured by the in-house radioimmunoassay approximately 4 months after they had been measured by the commercial kit method. Osteocalcin may have been degraded because of repeated freezing and thawing of the samples.

(ii) Patients with chronic renal failure are known to have "osteocalcin fragments" in their plasma (Gundberg and Weinstein, 1986) and these may be measured by one antibody and not by another. Worsfold et al, 1988, found an r value of 0.91 when they compared their radioimmunoassay with a commercial kit. However, their comparison was with samples from normal individuals and from patients with Paget's disease and patients with osteoporosis.

CHAPTER 4

STANDARD METHODS AND STATISTICAL TESTS

4.1. STANDARD METHODS

The standard methods and commercial kit methods used for the measurements made in this thesis are given below. Of the commercial kit methods used, the osteocalcin method was modified as described previously (Section 3.8.6); for all other methods the manufacturer's instructions were followed. Measurements described in Sections 4.1.7 to 4.1.10 were made by the technical staff of the Department of Clinical Chemistry, Royal Infirmary of Edinburgh.

4.1.1. ABO Blood groups

Anti-A and Anti-B blood grouping reagents were obtained from the Scottish National Blood Transfusion Service (BTS). Blood groups were determined by agglutination on whole blood.

4.1.2. Lewis groups

Anti-Lewis a and Anti-Lewis b reagents were obtained from BTS. The plasma was removed from a lithium heparin blood sample and the blood cells (20 μl volume) were washed with 0.15 mol/L NaCl. Saline (400 μl) was added to the cells. 20 μl of a saline suspension of red cells was added to an equal volume of Anti-Lewis a or Anti-Lewis b reagent in plastic tubes. After an overnight incubation at 4^{0}C, the samples were centrifuged at 500 xg for 1 minute and the presence or agglutination in the Anti-Lewis a or Anti-Lewis b tube was noted. In some samples, agglutination was not present in either tube and this sample was recorded as Lewis group $a^{-}b^{-}$.

4.1.3. Secretor status

A_1 mix, B and O washed red blood cell suspensions were obtained from BTS. Gorse lectin (Ulex europaeus) was from Sigma (product no. L-5505).

Secretor status was determined using specific inhibition of agglutination by saliva (Mollison, 1983). This method will detect the presence of H substance in saliva and thus define the secretor status of the individual. It will also act as a check on the blood group of secretors.

Dilutions of Anti-A, Anti-B and Anti-H (gorse lectin) were selected so that when mixed with A, B and O red cells respectively, agglutination occurred. In the case of Anti-A and Anti-B antisera, dilutions on either side of the selected dilution were also used. A typical series of dilutions used for Anti-A and Anti-B were 1:64, 1:128 and 1:256. Gorse lectin was used at dilutions of 1:8 and 1:16 of the stock lectin solution (100 mg/L in 0.01 M phosphate-buffered saline).

Saliva samples were centrifuged at 2,000 xg for 10 minutes and the supernatants boiled in a water bath for 20 minutes. Saliva (25 μl) was added to the diluted antisera in V-well microtitre plates. The plates were left at room temperature for at least 30 minutes when the appropriate blood was added. The plates were left overnight at 4°C and then examined for agglutination.

Table 4.1 summarises the agglutination patterns (+ = agglutination, - = no agglutination) found for non-secretors, and secretors of blood groups A, B, AB and O. In wells where agglutination was present, the saliva did not contain the relevant antigen.

Table 4.1. Agglutination patterns found for secretors and non-secretors.

Secretor Status	Well contents		
	Anti-A + A cells	Anti-B + B cells	Anti-H + O cells
Non-secretors	+	+	+
A secretors	-	+	-
B secretors	+	-	-
AB secretors	-	-	-
O secretors	+	+	-

4.1.4. Glutathione S-transferase

Plasma levels of glutathione S-transferase (GST) were measured by radioimmunoassay (Beckett and Hayes, 1984). Antiserum, standards and tracer were kindly provided by Dr G.J. Beckett, Department of Clinical Chemistry, The Royal Infirmary of Edinburgh. The antiserum used was specific for hepatic GST (B_1B_1) and showed no cross-reactivity with the anionic forms of GST found in red cells, muscle or lung. The between-batch CV for the measurement of GST was less than 9%.

4.1.5. Parathyroid hormone

Serum parathyroid hormone (PTH) was measured by the Allegro Intact PTH Kit (Nichols Institute; obtained from Biogenesis Ltd, Bournemouth, UK). This is a two-step immunoradiometric (IRMA) assay and measures the biologically-intact 84 amino acid chain of PTH. Two different goat polyclonal antibodies to human PTH are used. One antibody binds the mid-region and C-terminal PTH (39 - 84) and this antibody is immobilized onto plastic beads. The other antibody binds to N-terminal PTH 1 - 34 and this is labelled with [^{125}Iodine]. Serum for PTH measurement was separated and stored at -20^{0}C within 2 hours of collection of the blood. The between- batch CV for serum PTH measurement was 7%.

4.1.6. Osteocalcin

Plasma osteocalcin was measured using the Human Osteocalcin Radioimmunoassay Kit (OSTK-PR) from CIS UK Ltd, High Wycombe, Bucks, UK. Insufficient batches were run to determine between-batch precision for this method, although the manufacturers quote a figure of 6.8% at an osteocalcin concentration of 16.5 μg/L.

4.1.7. Free thyroxine and thyroid stimulating hormone

Free thyroxine (Free T_4) and thyroid stimulating hormone (TSH) were measured in plasma with the SimulTRAC Free T_4 [^{57}Co] / Thyroid Stimulating Hormone MAb [^{125}I] Solid Phase Component (Becton Dickinson, Orangeburg, New York). Free T_4 is measured by a competitive radioimmunoassay using a [^{57}Co] thyroxine derivative that does not bind significantly to thyroxine binding proteins. TSH is measured by a two-site IRMA, where a [^{125}I-labelled antibody reacts with TSH in the presence of a solid-phased second antibody. The between-batch CV for free T_4 measurement was 7% and for TSH measurement it was 5%.

4.1.8. Glycated haemoglobin

Glycated haemoglobin (HbA_1) was measured on haemolysed whole blood using commercially available agar plates (Corning: cat. no. 470055). Between-batch CV for HbA_1 measurement was 3.9%.

4.1.9. Measurements on the multi-channel analyser

The following measurements were carried out on plasma using the Sequential Multiple Analyser plus Computer (SMAC II: Technicon Instruments Corp., Basingstoke, UK): urea, creatinine, calcium, phosphate, albumin, bilirubin, alanine aminotransferase (ALT) and gamma-glutamyltransferase (GGT).

4.1.10. Magnesium

Plasma magnesium was measured on the RA-1000 (Technicon) by a colorimetric assay using the Wako kit (Wako Chemicals USA, Inc., Dallas, USA).

4.1.11. Aluminium

Plasma aluminium measurements were carried out by Mr J.Cowie, Medical Renal Unit, Royal Infirmary of Edinburgh. Aluminium was measured by atomic absorption.

4.1.12. 25-Hydroxyvitamin D

Measurements of serum 25-hydroxyvitamin D (25-OH-D) were performed by Dr N. Belton, Department of Child Life and Health, University of Edinburgh. A competitive binding assay which measures 25-hydroxycholecalciferol and 25-hydroxyergocalciferol was used (Preece et al, 1974). The between-batch CV for 25-OH-D measurement was 10%.

4.2. STATISTICAL TESTS

Standard parametric tests were used to compare group means (t-tests) and to measure correlations between variables. A Mann-Whitney test was used where the data were not normally distributed. The data were analysed with the use of the statistical package SPSS/PC+ on an IBM PS/2 Model 70.

CHAPTER 5

REFERENCE RANGES

This chapter concerns the establishment of reference ranges in plasma for the main forms of alkaline phosphatase and for osteocalcin, measured by the methods described previously (Chapters 2 and 3). Reference ranges for the other measurements carried out in this thesis (Chapter 4) will also be given.

5.1. Total, liver, bone and intestinal alkaline phosphatase

Two groups ("control" groups 1 and 2) were used to establish reference ranges for total, liver, bone and intestinal ALP. In the case of intestinal ALP, the activity was related to blood groups and secretor status. Measurements were carried out over the same time period as the clinical studies (control group 1 at the same time as the diabetic study - Chapter 6; control group 2 at the same time as the thyroid and renal studies - Chapters 7 and 8) so that results from patients could be compared directly with results from the corresponding control group.

(i) Control group 1 (hospital workers).
A venous blood sample and a saliva sample were collected from 44 healthy volunteers, all of whom were hospital workers. There were 18 males and 26 females in the group. The mean age was 35.6 years (SD = 11.1 years, range 20 - 63 years). The separated plasma and saliva were kept at -20^{o}C until analysis.

(ii) Control group 2 (blood donors).
Clotted blood samples from 100 blood donors were supplied by the Regional Blood Transfusion Service, Scotland. The age and sex of the donors was not documented. The separated serum was kept at -20^{o}C.

Liver and bone ALP were measured on all samples by the modified electrophoretic method. Intestinal ALP was measured by bromotetramisole-inhibition in control group 1 samples and by ELISA in control group 2 samples. ABO and Lewis blood groups were determined on whole blood. The secretor status was determined directly on saliva samples of control group 1. Secretor status of control group 2 individuals was inferred from the Lewis groups.

Total, liver and bone alkaline phosphatase.

Table 5.1 shows the mean, SD and range (U/L) of total, liver and bone ALP for control groups 1 and 2. There was minimal staining at the origin on polyacrylamide gel electrophoresis for the samples in both control groups. High-molecular-mass ALP, measured by ion-exchange in a random selection of samples with minimal staining at the origin, gave activities of less than 5 U/L.

Mean activities of total, liver and bone ALP were higher in control group 2 compared to control group 1 (Table 5.1). Possible reasons for this finding include a high proportion of young donors in control group 2 (resulting in a higher bone ALP) or the inclusion of individuals with undiagnosed liver disease among the donors (resulting in a higher liver ALP). Four of the donors had GGT activities above the reference range (55 U/L) and one had an ALT activity above the reference range (40 U/L), but these individuals all had liver ALP activities below 55 U/L. In the case of control group 1, two individuals had an ALT activity greater than 40 U/L and one had a GGT activity greater than 55 U/L; all three individuals had liver ALP activities less than 55 U/L.

Steinberg and Rogers, 1987, measured liver and bone ALP in the plasma of 67 adults by densitometric scanning of polyacrylamide gels. They found a mean liver ALP of 31.5 U/L (SD = 12.7 U/L) and a mean bone ALP of 26.5 U/L (SD = 12.7 U/L). Their means are similar to those obtained for control groups 1 and 2.

Table 5.1. Total, liver and bone ALP activities in control groups 1 and 2.

	Total ALP U/L	Liver ALP U/L	Bone ALP U/L
Control group 1			
Mean	57	20.6	29.0
SD	13.2	7.3	7.0
Range	35-91	8-45	17-45
Control group 2			
Mean	67	28.8	32.7
SD	21.8	13.3	11.7
Range	26-135	10-80	10-71

Intestinal ALP.

Table 5.2 shows intestinal ALP results for the control groups divided up into three categories:

Group B, O secretors	Lewis b
Group A secretors	Lewis b
Group A, B, O non-secretors	Lewis a

Individuals who were blood group AB secretors or Lewis a^-b^- (in the case of control group 2) were excluded.

Intestinal ALP measured by the BT method gave results which were slightly higher compared to those obtained by ELISA (Table 5.2). The ELISA method was previously shown to be more specific (Section 2.5). For both methods, no significant difference was found between intestinal ALP activities of A secretors and ABO non-secretors. BO secretors had significantly higher intestinal ALP activities ($p<0.05$ for both control groups) than A secretors or ABO non-secretors. This finding supports the original report by Langman et al, 1966, who showed that healthy adults, who were BO secretors, had a higher percentage of intestinal bands on electrophoresis compared to A secretors and non-secretors. Bailyes et al, 1988, measured intestinal ALP by an ELISA in 96 blood donors. They found a range of activities of 0.5 - 7.8 U/L for blood

Table 5.2. Intestinal ALP activities in plasma from control group 1 (measured by the bromotetramisole method) and control group 2 (measured by ELISA).

	Intestinal ALP (U/L)			
	All	BO secretors[a]	A secretors	Non-secretors
Control group 1				
n	44	18	11	15
Mean	7.9	13.0	4.6	4.1
SD	5.4	4.9	1.6	1.4
Range	2-25	5-25	2-8	2-6
Control group 2[b]				
n	100	44	23	28
Mean	5.9	9.1	3.0	2.9
SD	4.8	5.3	0.7	0.6
Range	2-23	3-23	2-4	2-4

[a] AB secretors not included.

[b] Lewis a^-b^- subjects not included.

groups A and AB and 0.7 - 14.3 U/L for blood groups O and B. It is not possible to compare their results directly with those in Table 5.2, since they did not take secretor status into account. However, intestinal ALP activities for the 100 blood donors ranged from 2 - 23 U/L (Table 5.2) while the range found by Bailyes et al, 1988, was narrower (0.5 - 14.3 U/L).

Table 5.3 summarises the reference ranges used for total, liver and bone ALP activities in plasma. These were obtained by taking cut-offs for activities which included 95% of the blood donor group (control group 2). The table also gives the upper limit for intestinal ALP activities expected for BO secretors and for non-secretors plus group A.

Table 5.3. Reference ranges for total, liver, bone, intestinal and high-molecular-mass ALP activities in plasma.

	Reference Range* (U/L)
Total ALP	40 - 110
Liver ALP	up to 55
Bone ALP	up to 55
Intestinal ALP	
BO secretors	up to 23
Non-secretors plus A	up to 4
High-molecular-mass ALP	up to 5

* derived from blood donor data (control group 2)

5.2. Osteocalcin

Blood was collected into lithium heparin tubes from 12 laboratory volunteers (9 males, 3 females; mean age 38 years, SD = 10.5 years, range 23 - 60 years). The plasma was separated and stored at - 20°C within 30 minutes of collection. Plasma samples from plasmapheresis donors were selected from routine laboratory specimens submitted to the Clinical Chemistry Department. The samples selected had been separated on the day of collection and had a total ALP activity within the reference range. Plasma samples were stored at -20°C until analysis. A total of 100 donor plasma samples were collected over several days. There were 60 males and 40 females in the group. The mean age was 35 years, SD = 9.0 years, range 20 - 62 years.

Osteocalcin was measured in all plasma samples using the in-house radio-immunoassay (Section 3.8). In 15 of the plasma samples, osteocalcin was measured using radioimmunoassay but with delayed tracer addition. In patients (e.g. diabetics, Chapter 6) where possible low or low/normal osteocalcin concentrations were being investigated, osteocalcin concentrations in patients (also measured by

radioimmunoassay with delayed tracer addition) were compared to those obtained in these 15 plasma samples.

The mean osteocalcin concentration for the laboratory volunteers was similar to that obtained for the blood donors (Table 5.4). The use of delayed tracer addition in 15 of the plasma samples gave a lower mean (mean osteocalcin = 4.7 μg/L, SD = 1.9 μg/L) and narrower range of osteocalcin concentrations (range 1.9 - 9.6 μg/L) compared to the radioimmunoassay where antibody and tracer were added at the same time.

Within the plasmapheresis donor group, plasma osteocalcin of males did not differ significantly from that of females. Osteocalcin showed a significant negative correlation with age ($p<0.001$; $r= -0.37$). The upper reference limit (mean + 2SD) for osteocalcin from the donor data was 11.5 μg/L.

The mean osteocalcin concentration obtained in the present study was similar to that reported for normal individuals with other radioimmunoassays [Price et al, 1980: 4.5 μg/L (SD 2.0 μg/L); Gundberg et al, 1985: 7.0 μg/L (range 3 - 13 μg/L); Johansen et al, 1987: 7.4 μg/L (SD 2.5 μg/L)]. The commercial kit insert (CIS UK Ltd) quotes a mean of 6.7 μg/L (SD 2.8 μg/L) for males and 4.7 μg/L (SD 2.2 μg/L) for females.

Table 5.4. Plasma osteocalcin concentrations in laboratory volunteers and plasmapheresis donors.

Group	Osteocalcin (μg/L)			
	n	Mean	SD	Range
Laboratory volunteers	12	6.8	2.4	3.2-10.8
Plasmapheresis donors				
Males	60	6.2	2.8	1.3-16.4
Females	40	5.3	2.8	1.0-12.6
All	100	5.8	2.9	1.0-16

5.3. Other measurements

Reference ranges relating to healthy adults for the other analytes measured in this thesis are given in Table 5.5. In most cases, the reference ranges quoted in the Laboratory Handbook of the Department of Clinical Chemistry, Royal Infirmary of Edinburgh were used. The reference range for plasma GST, obtained from the concentration of GST in blood donors, was provided by Dr G.J. Beckett. The reference range for serum 25-OH-D was provided by Dr N. Belton and was based on 25-OH-D levels in healthy adults measured in early winter.

Table 5.5. Reference ranges for analytes measured in this thesis.

Analyte	Reference Range
Bilirubin	2-17 μmol/L
ALT	10-40 U/L
GGT	
males	10-55 U/L
females	5-35 U/L
GST	<4.5 μg/L
Phosphate	0.8-1.4 mmol/L
Calcium	2.12-2.62 mmol/L
Magnesium	0.75-1.0 mmol/L
Aluminium	<0.4 μmol/L
Urea	2.5-6.6 mmol/L
Creatinine	55-150 μmol/L
Albumin	36-47 g/L
Glycated haemoglobin	4.5-8.0%
PTH	10-55 ng/L
TSH	0.3-5.0 mU/L
Free T_4	9-23 pmol/L
25-OH-D	>10 μg/L

CHAPTER 6

PLASMA ALKALINE PHOSPHATASE FORMS IN DIABETES MELLITUS

6.1. Introduction

Increases in plasma total ALP activity have been reported in up to 44% of patients with uncomplicated diabetes (Belfiore et al, 1973). However, Goldberg et al, 1977, found a much lower percentage of diabetics with an elevated serum ALP (11% of out-patients and 17% of in-patients). There has also been controversy regarding the source of the elevated ALP. Some workers (Foster et al, 1980; Salmela et al, 1984) have found a high occurrence of abnormal liver function tests in diabetes suggesting a liver source for the raised total ALP. Stepan et al, 1980, measured ALP forms by an inactivation-inhibition method in type 1 and type 2 diabetics. They found that the increase in total ALP activity was due to the bone isoenzyme in 39% of their patients. In 19% of those cases, there was a simultaneous increase in the liver isoenzyme. Maxwell et al, 1986, carried out qualitative agarose gel electrophoresis of ALP isoenzymes to investigate the source of raised ALP in their series of patients. They found that the bone fraction was the dominant band in all patients.

Recently, serum osteocalcin has been used as a specific marker of bone formation in diabetics (Pietschmann et al, 1988). Osteocalcin was shown to be significantly lower in type 2 diabetics compared to control subjects. Osteocalcin levels of type 1 diabetics were not significantly different from control subjects but, within the group, levels were significantly lower in patients with retinopathy and/or proteinuria compared to patients without microangiopathy.

Skillen et al, 1982, measured intestinal ALP by bromotetramisole-inhibition in insulin-dependent and maturity-onset diabetics. They found a raised intestinal ALP activity in 28% of insulin-dependent diabetics although in maturity-onset diabetics intestinal ALP activity was not significantly different to a control group of out-patients. In their study, total ALP activities of the diabetics were within the reference range, showing that an abnormal isoenzyme pattern can be present with a normal total ALP activity.

The aims of the present study were:

1. To determine the frequency and extent of increase in plasma total ALP activity in type 1 and type 2 diabetics.
2. To quantitate liver, bone and intestinal ALP in the plasma of diabetics and to compare the levels to those in healthy volunteers.
3. To relate liver ALP activity to other "liver function tests" (bilirubin, ALT and GGT) and bone ALP activity to osteocalcin concentrations in type 1 and type 2 diabetics.

6.2. Patients and controls

Patients were recruited from those routinely attending the Diabetic Department, The Royal Infirmary of Edinburgh; the first 5 to 12 out-patients at each morning clinic were asked to participate. Patients who had type 1 disease were classified by insulin dependence, their clinical history and family history of the disease. The patients who had type 2 disease were not dependent on insulin. The control group used was described in Section 5.1 (control group 1) and was made up of 44 hospital workers. Details of the diabetic and control group are given in Table 6.1.

6.3. Samples

A non-fasting venous blood sample and a saliva sample were collected from each patient and control. The time of sampling was mid-morning in all cases. Samples were collected from the diabetics and controls over the same time period. A portion of whole blood was kept at 4°C. The separated plasma and saliva were kept at -20°C until analysis.

Additional plasma samples from diabetics were selected from routine laboratory specimens and stored at -20°C within 4 hours of collection. This additional group (11 males, 9 females; mean age = 52.3 years, SD = 13.6 years, range 28 - 79 years) consisted of 8 type 1 diabetics and 12 type 2 diabetics. Osteocalcin concentrations in this additional group were compared to those found in 15 plasmapheresis donors (9 males, 6 females; mean age = 32.6 years, SD = 6.2 years, range 24 - 44 years) which were described previously (Section 5.2).

Table 6.1. Details of the diabetic and control group.

	Type 1	Type 2	Controls
Number			
Total	83	83	44
Male	48	49	18
Female	35	34	26
Age (years)			
Mean	39.5	56.0	35.6
SD	13.3	6.8	11.1
Range	20-65	38-65	20-63
Duration of diabetes (years)			
Mean	7.1	11.7	-
SD	5.8	8.2	-
Range	1-22	1-45	-

6.4. Measurements

The following measurements were made:

On plasma samples: Total ALP; liver and bone ALP (by the modified electrophoretic method); intestinal ALP (by bromotetramisole-inhibition); bilirubin, ALT and GGT; osteocalcin (on the additional sample group, by the in-house radioimmunoassay with delayed tracer addition).

On whole blood: ABO and Lewis blood groups; glycated haemoglobin.

On saliva: secretor status.

6.5. Results

Mean HbA_1 of type 1 diabetics was 10.9% (SD = 2.2%, range 6.6 - 17.5%) and of type 2 diabetics 10.3% (SD = 2.7%, range 6.1 - 20.7%). The numbers of diabetics and controls with bilirubin, ALT, GGT and total ALP above the reference range are given in Table 6.2.

Table 6.2. Number (percentage) of diabetics and of controls with results for bilirubin, ALT, GGT and total ALP above the reference range.

	n	Bilirubin	ALT	GGT	Total ALP
Type 1	83	8(9.5)	3(3.5)	4(5.0)	7(8.5)
Type 2	83	6(7.0)	18(21.5)	26(31.5)	10(12.0)
Controls	44	2(4.5)	2(4.5)	1(2.5)	0(0)

Tables 6.3 and 6.4 show the mean, SD and range of total ALP and intestinal ALP activities in the diabetics and controls, categorised by blood group/secretor status. Figure 6.1 shows the data for intestinal ALP.

Table 6.3. Total ALP activity of type 1, type 2 diabetics and the control group[a].

	Total ALP (U/L)		
	BO secretors	A secretors	Non-secretors
Type 1			
n	35	15	33
Mean	79.5	67.7*	59.7
SD	24.4	20.8	15.5
Range	48-162	32-109	39-92
Type 2			
n	36	23	24
Mean	85.9*	65.0**	70.7*
SD	32.1	11.6	21.3
Range	40-137	47-86	42-128
Controls			
n	18	11	15
Mean	67.4	48.1	51.9
SD	12.0	7.8	15.7
Range	36-91	35-62	35-69

[a]The diabetics (type 1 or 2) were compared with the controls within each blood/group secretor status category. Asterisks show the level of significance (*$p<0.01$, **$p<0.001$).

Table 6.4. Intestinal ALP activity of type 1, type 2 diabetics and the control group[a].

	Intestinal ALP (U/L)		
	BO secretors	A secretors	Non-secretors
Type 1			
n	35	15	33
Mean	27.2**	9.3**	8.2**
SD	13.5	3.5	3.9
Range	9-64	3-19	3-19
Type 2			
n	36	23	24
Mean	30.0**	8.6**	9.3**
SD	22.2	2.7	3.7
Range	7-106	5-15	5-20
Controls			
n	18	11	15
Mean	13.0	4.6	4.1
SD	4.9	1.6	1.4
Range	5-25	2-8	2-6

[a]The diabetics (type 1 or 2) were compared with the controls within each blood group/secretor status category. Asterisks indicate the level of significance (**$p<0.001$).

Within all groups of patients and within the control group, there was no significant difference in either total ALP or intestinal ALP between A secretors and ABO non-secretors. On the other hand, within each diabetic group and within the control group, both total ALP and intestinal ALP were significantly higher in BO secretors than in A secretors and ABO non-secretors ($p<0.05$ in all cases). The activities of intestinal ALP of BO secretors of type 1 and type 2 diabetics did not differ significantly from one another but both were significantly higher than BO secretors of the control group ($p<0.001$). Similarly, A secretors and ABO non-secretors of the diabetics had significantly higher intestinal ALP activity than A secretors and ABO non-secretors of the control group ($p<0.001$).

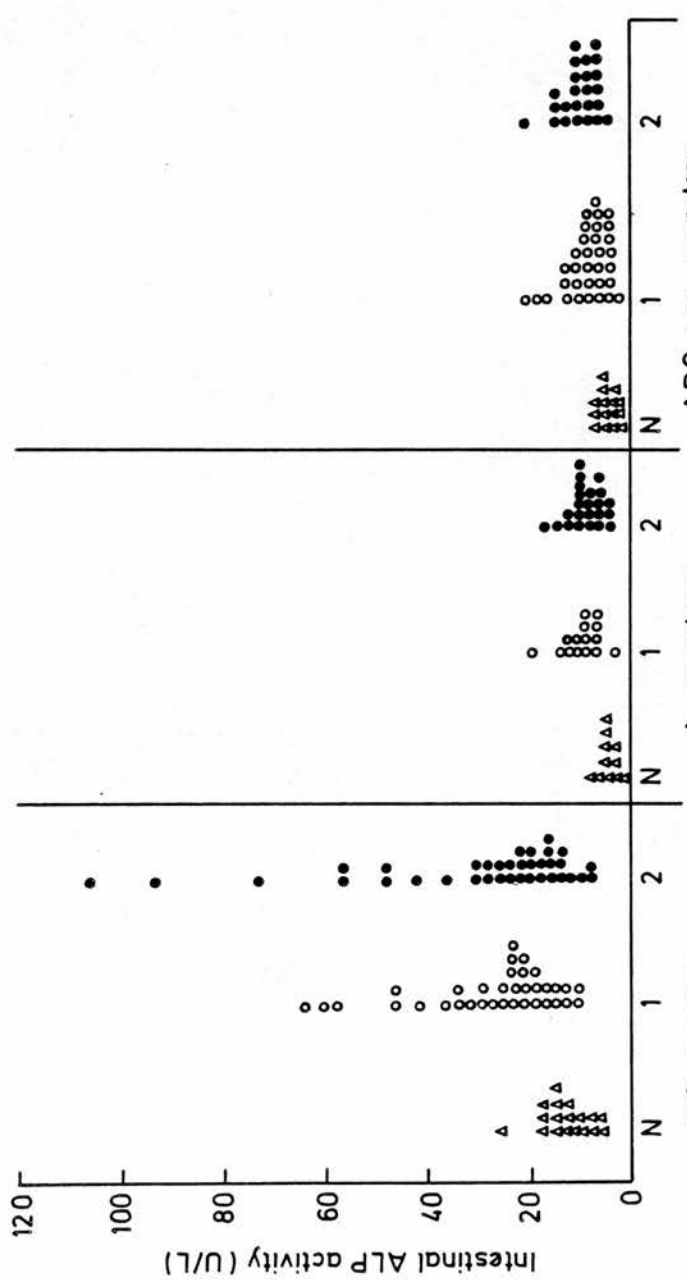


Figure 6.1. Plasma intestinal ALP activity in the control group (N - Δ), type 1 diabetics (1 - o) and type 2 diabetics (2 - ●) for the three blood group/secretor status categories (BO secretors, A secretors and ABO non-secretors).

Intestinal ALP activities given so far in this study were in non-fasting plasma samples. It is known that intestinal ALP activity increases in plasma after fat ingestion (Kleerekoper et al, 1970). It was therefore decided to measure intestinal ALP in fasting samples to see if this resulted in a reduction in the level of intestinal ALP activity. For this, eight diabetics (all O secretors) with intestinal ALP activity greater than 40 U/L were selected. Fasting samples were taken from these patients at the next clinic visit. In all cases, intestinal ALP was significantly lower ($p<0.01$) in the fasting compared to the non-fasting sample (mean fasting intestinal ALP = 31.1 U/L, SD = 20.9 U/L, range = 11-79 U/L; mean non-fasting intestinal ALP = 63.3 U/L, SD = 24.9 U/L, range = 41 - 106 U/L). Intestinal ALP activity of the non-fasting and fasting samples of the eight diabetics are given in Figure 6.2. The dotted lines show the range of intestinal ALP activity found for BO secretors of the control group. In most cases, the fasting sample from the diabetics had a higher intestinal ALP than was found in the samples from the control group.

Table 6.5 shows the mean, SD and range of liver and bone ALP activities of the diabetics and the control groups. Both type 1 and type 2 diabetics had significantly higher liver ALP activities than the control group. Type 2 diabetics had higher liver ALP activities than type 1 diabetics ($p<0.01$). Five diabetics had plasma liver ALP activities greater than 55 U/L (liver ALP activities = 78 U/L, 66 U/L, 59 U/L, 57 U/L and 56 U/L), one of these had a raised plasma GGT activity. High-molecular-mass ALP was not measured in this study since all samples showed minimal staining at the origin on polyacrylamide gel electrophoresis, indicating high-molecular-mass ALP activities of less than 5 U/L in these samples.

There was no significant difference between bone ALP activities of type 1, type 2 diabetics and the control group (Table 6.5). Bone ALP activity was greater than 55 U/L in two diabetics, and in one of these it accounted for most of the increase in total ALP activity above the reference range. In the other, both bone ALP and intestinal ALP contributed to the raised total ALP activity.

Figure 6.2. Intestinal ALP activity of non-fasting and fasting plasma samples from 8 diabetics. The dotted lines show the range of intestinal ALP activity found in BO secretors of the control group.

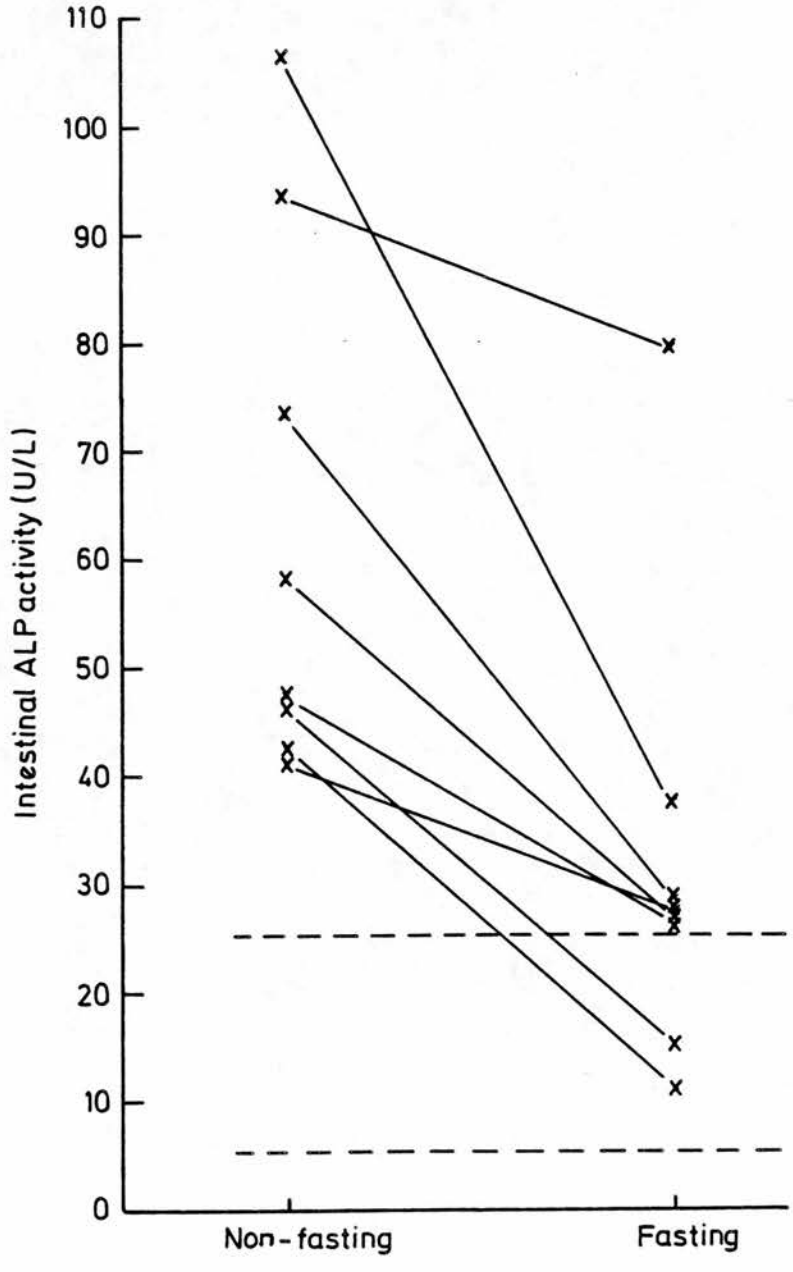


Table 6.5. Liver and bone ALP activity of type 1, type 2 diabetics and of the control group[a].

	Liver ALP (U/L)	Bone ALP(U/L)
Type 1		
n	83	83
Mean	25.9*	27.1
SD	13.5	8.6
Range	8-78	15-52
Type 2		
n	83	83
Mean	29.0**	28.7
SD	10.7	11.1
Range	11-59	14-63
Controls		
n	44	44
Mean	20.6	29.0
SD	7.3	7.0
Range	8-45	17-45

[a]The diabetics (type 1 or 2) were compared with the controls. Asterisks indicate the level of significance (*$p<0.05$, **$p<0.001$).

Type 1 and type 2 diabetics were grouped together for the analysis of osteocalcin data on the additional sample group, since numbers were too small for consideration of each type of diabetes separately. Mean osteocalcin concentration in the diabetics (3.5 μg/L, SD = 1.9 μg/L, range 0.9 - 7.8 μg/L) was not significantly different from mean osteocalcin in the plasmapheresis samples (mean osteocalcin = 4.7 μg/L, SD = 1.9 μg/L, range 1.9 - 9.6 μg/L).

6.6. Discussion

Total ALP was raised in 8.5% of type 1 diabetics and 12% of type 2 diabetics (Table 6.2). Goldberg et al, 1977, found a similar percentage with a raised total ALP (11%) in their series of diabetic out-patients. The abnormalities in ALP isoenzymes found in the present study related mainly to intestinal ALP with minor abnormalities in liver and bone ALP. This finding is different to other reports (Stepan et al, 1980; Maxwell et al, 1986) where abnormalities in bone ALP were shown to be the most important.

The pattern of higher intestinal ALP activities in BO secretors compared to A secretors and ABO non-secretors shown for the control group was also found in type 1 and type 2 diabetics (Figure 6.1). The association of plasma intestinal ALP activities with blood group and secretor status was first demonstrated in healthy adults by Langman et al, 1966. It has also been found in patients with cirrhosis (Stolbach et al, 1967) but has not been reported previously in diabetics.

Of more importance, the results show that, within each blood group/secretor status category, intestinal ALP was significantly higher in diabetics than in the non-diabetic control group (Figure 6.1). The results also show that there was no significant difference between type 1 and type 2 diabetics when they were compared for each blood group/secretor status category. Skillen et al, 1982, reported a raised intestinal ALP activity in diabetes. However, in their study, the increased activity was confined to type 1 diabetics.

The magnitude of the increased activity of intestinal ALP (up to 69% of total ALP activity in one patient) was sufficient to explain the raised plasma ALP activity in 11 out of 17 patients with raised total ALP activity. These patients were all BO secretors. Therefore, in a non-fasting sample, intestinal ALP is an important source of raised total ALP activity in diabetics who are BO secretors.

The reason for the increased plasma intestinal ALP activity in diabetes is unclear. The use of a fasting sample resulted in a reduction in plasma intestinal ALP activity (Figure 6.2) although levels found in the fasting sample were, in most cases, higher than those of BO secretors of the control group. Intestinal ALP increases in the

thoracic duct and in plasma after ingestion of a fatty meal (Kleerekoper at al, 1970). It may be that diabetics have an abnormal response to a fat load with an exaggerated rise in plasma intestinal ALP activity.

Stolbach et al, 1967, reported a raised intestinal ALP in patients with cirrhosis; this was thought to be the result of impaired clearance of the enzyme. Six of the 14 patients with intestinal ALP activity greater than 40 U/L had an associated abnormality of GGT or ALT. Disturbed liver function may explain the raised intestinal ALP in some of the diabetics.

In healthy rats, the majority of serum intestinal ALP is of intestinal origin (Saini and Posen, 1969). Diabetic rats have significantly higher serum intestinal ALP activities than normal rats (Chua and Shrago, 1978). Insulin administration restores serum ALP activity of the diabetic rat to normal levels (Hough et al, 1981). Although it is not possible to relate the results of animal experiments directly to human diabetes, insulin deficiency may contribute to the raised intestinal ALP activity demonstrated in this study.

Liver ALP in type 1 and type 2 diabetes was higher than in the control group. Type 2 diabetics had significantly higher liver ALP activities compared to type 1 diabetics (Table 6.5). There was also a greater number of abnormal results for ALT and GGT in type 2 diabetics compared to type 1 diabetics (Table 6.2). Both these findings suggest a greater incidence of liver disease in type 2 than in type 1 diabetics. Salmela et al, 1984, found an increased plasma GGT activity in 19% and an increased ALT activity in 17% of out-patients. They also demonstrated a greater incidence of abnormal liver function tests in non-insulin dependent diabetics.

Bone ALP was not significantly different in the three groups (Table 6.5). Bone ALP accounted for most of the increase in total ALP above the reference range in only 2 diabetics. This differs from Stepan et al, 1980, who reported an increase in total ALP activity because of an increased bone ALP in 39% of their series of diabetic patients. The finding of a plasma osteocalcin concentration in the additional diabetic group which did not differ from the blood donor group supports the data for bone ALP activity. Both bone ALP and osteocalcin are markers of osteoblast function and would be expected to behave in a similar fashion. In a study carried out by Pietschmann et

al, 1988, serum levels of osteocalcin and 25-hydroxyvitamin D levels were found to be significantly lower in type 2 diabetics compared to an age-matched control group. They concluded that there was evidence of decreased bone formation in patients with type 2 diabetes mellitus. The numbers in the additional study were too small to show up any difference; osteocalcin measurements in a larger group of diabetics and in an age-matched control group are needed to investigate this further.

CHAPTER 7

PLASMA ALKALINE PHOSPHATASE FORMS IN HYPERTHYROIDISM

7.1. Introduction

Both bone and liver abnormalities have been described in patients with hyperthyroidism. Clinical manifestations of the poorly understood osteodystrophy of hyperthyroidism are rare although osteoporosis can sometimes occur. Mundy et al, 1976, have shown that thyroid hormones have a direct positive effect on bone resorption independent of PTH, vitamin D and prostaglandins. Increased bone resorption results in increased plasma calcium concentrations and increased urinary calcium and hydroxyproline excretion (Mosekilde et al, 1978). In addition to increased osteoclastic activity, there is in vitro evidence that thyroid hormones stimulate osteoblastic cells with increased production of ALP (Sato et al, 1987). Both total ALP and osteocalcin have been reported to be increased in the plasma of hyperthyroid patients (Garrel et al, 1986; Lukert et al, 1986).

Evidence of the presence of subclinical liver disease in hyperthyroidism comes from reports of abnormal bromosulphthalein retention times and elevations of bilirubin and liver enzymes in these patients (Ashkar et al, 1971; Azizi, 1982; Beckett et al, 1985b). Electron microscopy studies of liver biopsies from hyperthyroid patients have shown enlarged mitochondria, hypertrophy of smooth endoplasmic reticulum and reduced glycogen stores (Klion et al, 1971). These histological findings reflect the relative hypoxia of hepatocytes in the thyrotoxic state, where oxygen consumption is increased (Sheridan, 1983).

Raised total ALP activities in the plasma of hyperthyroid patients have been reported by Cassar and Joseph, 1969, Cooper et al, 1979, and Rhone et al, 1980. These studies found raised total ALP activities in 37% to 89% of hyperthyroid patients. Rhone et al, 1980, also gave information on the tissue source of ALP in hyperthyroid patients: liver ALP was elevated in 63% and bone ALP in 68% of patients. Quantitative data for intestinal ALP was not given.

The aims of this study on hyperthyroid patients were:

1. To measure total, liver, bone and intestinal ALP activities in a group of hyperthyroid patients and to compare the activities with those in treated thyrotoxicosis and blood donors.
2. To correlate the findings for liver ALP with other markers of liver disease (bilirubin, ALT, GGT and GST).
3. To correlate the findings for bone ALP with other markers of bone disease (calcium, PTH and osteocalcin).

7.2. Patients

Group 1. Hyperthyroid patients.

Patients in this group were attending the Thyroid Clinic for investigation of thyroid disease. Patients who were clinically and biochemically hyperthyroid (plasma TSH <0.1 mU/L, free T_4 >24 pmol/L) were selected.

Group 2. Euthyroid patients (treated thyrotoxicosis).

The patients in this group had been previously thyrotoxic but at the time of sampling were clinically and biochemically euthyroid (plasma TSH and free T_4 within the reference range). One patient had exophthalmic Graves' disease. Treatment consisted of radioactive iodine (n = 35), thyroidectomy (n = 15) or carbimazole (n = 7). The mean time since treatment was 6.5 years (SD = 5.9 years, range 0.5 - 32 years). Twenty seven patients were taking T_4; the remainder were on no replacement therapy. Patient details are given in Table 7.1.

7.3. Measurements

On plasma: total ALP; liver and bone ALP; intestinal ALP (by ELISA); osteocalcin (by the in-house radioimmunoassay); bilirubin, ALT, GGT and GST; calcium and albumin; TSH and free T_4.

On serum: PTH (measured in 46 of group 1 patients and 39 of group 2 patients).

On whole blood: ABO and Lewis groups.

On saliva: secretor status.

Total, liver, bone and intestinal ALP activities in hyperthyroid (group 1) and euthyroid (group 2) patients were compared to those found in serum samples from 100 donors (control group 2 - Section 5.1). Osteocalcin concentrations in hyperthyroid and euthyroid patients were compared to those found in plasma of plasmapheresis donors (Section 5.2).

Table 7.1. Age and sex distribution of patients in group 1 (hyperthyroid) and group 2 (euthyroid).

	Group 1	Group 2
Number		
Total	63	58
Male	10	10
Female	53	48
Age (years)		
Mean	52	47
SD	13.4	15.1
Range	22-85	19-81

7.4. Results

Thyroid function tests.

Patients in group 1 had TSH concentrations <0.1 mU/L and a mean free T_4 of 56 pmol/L (SD = 24.9 pmol/L, range 24 - 123 pmol/L). Group 2 patients had a mean TSH of 1.9 mU/L (SD = 1.3 mU/L, range 0.3 - 4.9 mU/L) and a mean free T_4 of 15 pmol/L (SD = 3.9 pmol/L, range 9 - 24 pmol/L).

Total alkaline phosphatase and alkaline phosphatase forms.

All the samples in group 1 and 2 showed minimal staining at the origin on polyacrylamide gel electrophoresis, indicating a high-molecular-mass ALP activity of less than 5 U/L in these samples. Total, liver and bone ALP activities were all higher in

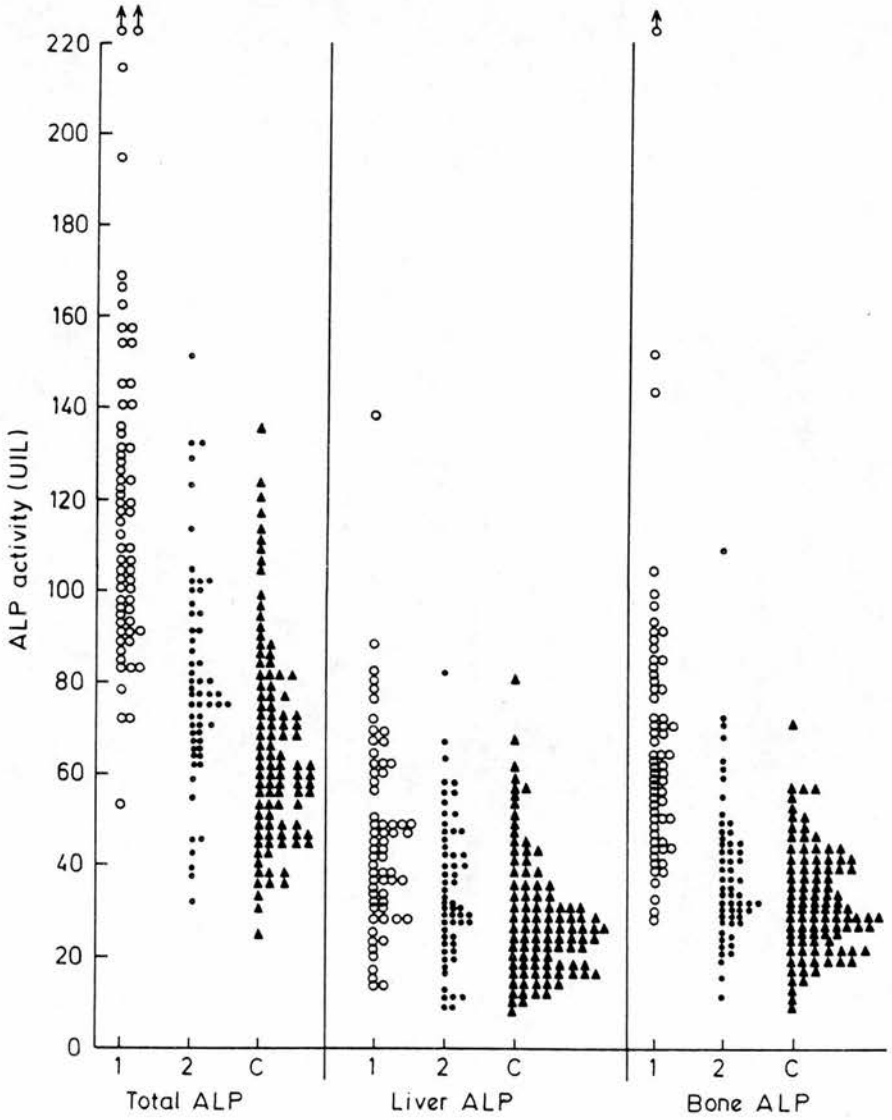
group 1 compared to group 2 ($p<0.001$ in all cases) (Table 7.2). Group 2 also showed higher activities for total ALP ($p<0.005$), bone ALP ($p<0.02$) and liver ALP ($p<0.01$) than did the control group. There was no correlation between free T_4 and total, liver or bone ALP in either group 1 or 2. Figure 7.1 shows the individual values obtained for total, liver and bone ALP in the patient and control groups.

Table 7.2. Total, liver and bone ALP of group 1, group 2 and the controls.

	Total ALP U/L	Liver ALP U/L	Bone ALP U/L
Group 1			
Mean	124.0	46.9	69.3
SD	51.5	22.0	42.1
Range	53-407	14-138	28-340
Group 2			
Mean	80.0	35.4	38.7
SD	24.1	15.7	16.4
Range	32-151	9-82	10-109
Controls			
Mean	67.4	28.8	32.7
SD	21.8	13.3	11.7
Range	26-135	10-80	10-71

Intestinal ALP activities were related to the three blood group/secretor status categories (BO secretors, A secretors and non-secretors)(Table 7.3). Within each patient group and the control group, higher activities were found in BO secretors than in A secretors and non-secretors. Comparing the three groups, there was no significant difference found in intestinal ALP activity of group 1, group 2 or the controls, either when all categories were considered together or when BO secretors were considered alone. Group A secretors and non-secretors of group 1 and group 2 showed higher activities of intestinal ALP than did the control group.

Figure 7.1. Total, liver and bone ALP activities in group 1 (o), group 2 (●) and the control group (▲).



Within the treated thyrotoxic group, there was no correlation between total, liver, bone or intestinal ALP and the time since treatment of thyrotoxicosis. Patients treated with carbimazole had significantly lower ($p<0.005$) total and liver ALP activities than did patients treated with radioactive iodine. This may reflect the different ages of the patients within each treatment category (patients under the age of 40 years generally receive carbimazole while those over 40 years are usually treated with radioactive iodine) with a greater incidence of liver abnormalities in the older age group. There was no correlation between ALP variables (total, liver, bone or intestinal ALP) and thyroxine dose.

Table 7.3. Intestinal ALP activities of group 1, group 2 and the controls.

	Intestinal ALP (U/L)			
	All	BO secretors[a]	A secretors	Non-secretors
Group 1				
n	63	28	15	20
Mean	7.3	11.5	4.0	3.7
SD	5.0	4.6	1.5	0.9
Range	3-22	5-22	3-8	3-7
Group 2				
n	58	19	16	21
Mean	6.3	10.0	4.4	3.8
SD	4.3	5.0	1.4	0.7
Range	2-23	3-23	2-7	3-6
Controls[b]				
n	100	44	23	28
Mean	5.9	9.1	3.0	2.9
SD	4.8	5.3	0.7	0.6
Range	2-23	3-23	2-4	2-4

[a]AB secretors not included.

[b]Lewis a$^-$b$^-$ not included.

Liver-related measurements.

Mean plasma levels for bilirubin, ALT, GGT and GST for group 1 patients were significantly higher ($p<0.05$ in all cases) than in group 2 patients (Table 7.4). There was a positive correlation between free T_4 and GST in group 1 patients but no correlation between free T_4 and bilirubin, ALT or GGT.

Table 7.4. Bilirubin, ALT, GGT and GST in group 1 and group 2 patients.

	Bilirubin μmol/L	ALT U/L	GGT U/L	GST μg/L
Group 1				
Mean	9.8	32	36	5.4
SD	3.5	16.9	35.5	3.1
Range	6-22	10-102	6-217	1-19
Group 2				
Mean	6.9	18	16	2.7
SD	2.7	6.5	10.6	1.3
Range	3-12	10-37	5-63	1-6

Bone-related measurements.

Group 1 had higher concentrations of calcium and lower albumin concentrations in plasma compared to group 2 patients (Table 7.5). The differences were all statistically significant ($p<0.05$). The differences between the two groups in ionized calcium is probably greater than the difference in total calcium because albumin concentrations in group 1 were lower than those in group 2.

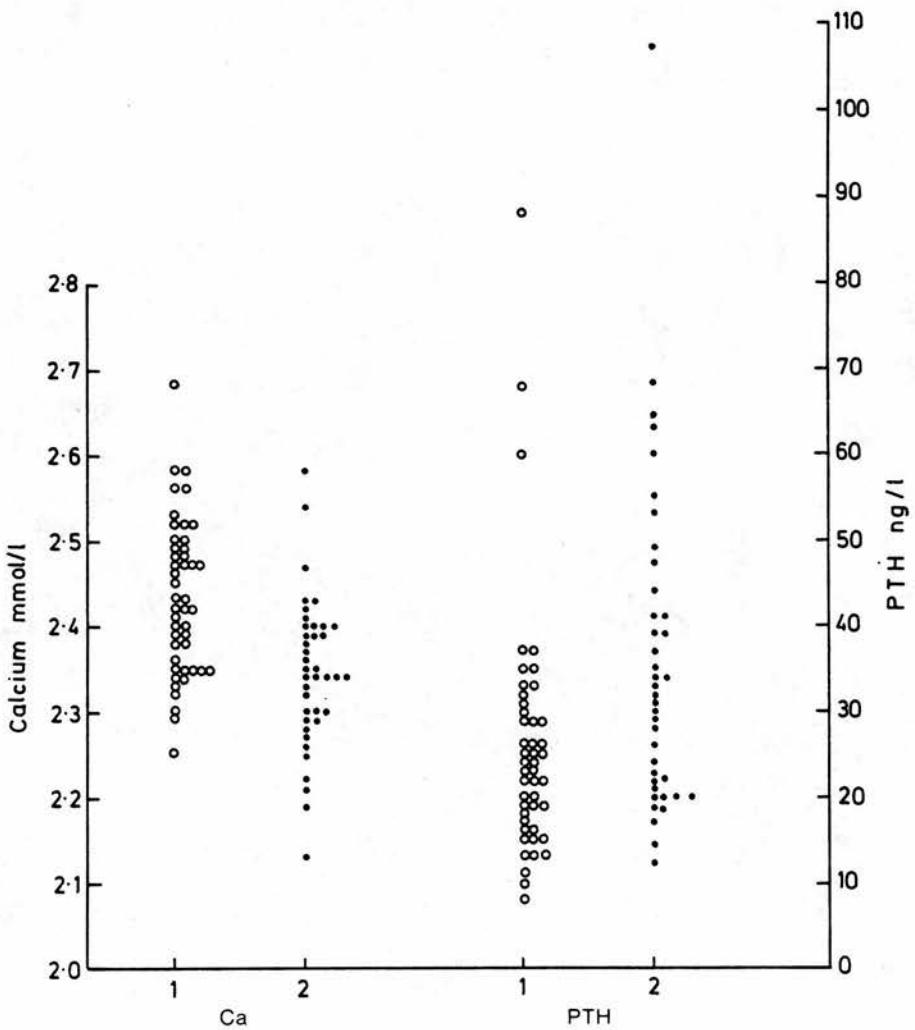
Figure 7.2 shows the concentration of calcium in plasma and of PTH in serum from group 1 and group 2 patients. The higher mean calcium in group 1 was associated with a significantly lower mean PTH in group 1 than in group 2 (Table 7.5). Patients with PTH concentrations greater than 55 ng/L had plasma calcium and plasma creatinine concentrations within the reference range.

Plasma osteocalcin concentrations of group 1 patients were significantly higher ($p<0.001$) than those of group 2 patients (Table 7.5). Within each group, plasma osteocalcin correlated with total ALP activity (group 1, $r = 0.50$; group 2, $r = 0.42$) and more closely with bone ALP activity (group 1, $r = 0.61$; group 2, $r = 0.71$). Group 1 had significantly higher ($p<0.001$) osteocalcin levels compared to the plasmapheresis donor group (Section 5.2) while osteocalcin levels in group 2 were not significantly different to those in the plasmapheresis donor group. There was no correlation between free T_4 and any of the bone-related measurements (calcium, PTH or osteocalcin).

Table 7.5. Calcium, albumin, PTH and osteocalcin in group 1 and group 2.

	Calcium mmol/L	Albumin g/L	PTH ng/L	Osteocalcin µg/L
Group 1				
Mean	2.44	43	25.9	14.0
SD	0.09	2.8	14.7	7.9
Range	2.29-2.68	37-48	8-88	1-52
Group 2				
Mean	2.33	45	35.6	6.7
SD	0.09	2.1	18.8	4.2
Range	2.09-2.58	40-49	12-107	1-24

Figure 7.2. Plasma calcium and serum PTH in group 1 (o) and group 2 (•) patients.



7.5. Discussion

Total ALP activity was increased above the reference range (>110 U/L) in 51% of untreated hyperthyroid patients. This compares well with the study of Cooper et al, 1979, who found ALP increases in almost half of their series of thyrotoxic patients. However, Rhone et al, 1980, found 77% of thyrotoxic patients had ALP activities greater than 110 U/L. Total ALP activity of group 1 patients did not correlate with free T_4. This contrasts with other studies (Cooper et al, 1979; Martinez et al, 1986) where significant correlations were found between total ALP and total or free T_4.

Liver ALP activity was raised (>55 U/L) in 30% of untreated hyperthyroid patients (group 1). Mean liver ALP activity of group 1 was significantly higher than that of the treated thyrotoxic group (group 2) (Table 7.2). Rhone et al, 1980, found that 63% of those in their study of thyrotoxic patients had liver ALP activities above those of control subjects.

The lower percentage of patients with abnormal liver ALP activities in the present study compared to the study of Rhone et al, 1980, may reflect a difference in the severity of thyrotoxicosis. It may also be the result of the different methods used in the two studies. Rhone et al, 1980, separated liver and bone ALP on cellulose acetate and quantified the bands by densitometric scanning. Separation of ALP forms on cellulose acetate is not as clear as on polyacrylamide (Moss, 1986b). An elevated bone ALP (found in 68% of patients studied by Rhone et al, 1980) may have, because of poor separation, led to a falsely elevated liver ALP. A raised bone ALP was previously shown not to interfere in liver ALP measurement by the modified electrophoretic method (Section 2.4.5).

Indices of liver disease other than liver ALP (bilirubin, ALT, GGT and GST) were also higher in group 1 compared to group 2 (Table 7.4). Other workers have found ALT activities within the reference range (Cooper et al, 1979), increased GGT activities (Azizi, 1982) and increased GST levels (Beckett et al, 1985b) in hyperthyroidism. Of the "liver function tests" measured in this study, only GST had a mean level above the reference range in group 1 patients. Plasma GST is known to be more sensitive at detecting liver damage than the aminotransferases (Beckett et al, 1985a). The

significant correlation between GST and free T_4 indicates that the extent of liver dysfunction is related to the severity of the thyrotoxicosis.

Therefore, patients with untreated hyperthyroidism show evidence of liver dysfunction although in most cases this is minor. Comparison with group 2 patients suggests that the abnormalities largely resolve on treatment of hyperthyroidism. Although the euthyroid group had significantly higher activities of liver ALP than did the control group (Table 7.2), the range and distribution of activities in the two groups were similar (Figure 7.1).

Bone ALP activities were greater than 55 U/L in 63% of hyperthyroid patients. This compares well with the results of Rhone et al, 1980, who found that 68% of their patients had activities exceeding those in control sera. The range and distribution of bone ALP activities in group 2 were similar to those in the control group (Figure 7.1), apart from one patient, who had been treated with radioactive iodine six months previously, whose bone ALP activity was 108 U/L. The high bone ALP activity in this case is consistent with the findings of Cooper et al, 1979, who showed an increase in ALP activity (predominantly of bone origin) after treatment with radioactive iodine, with a peak activity at 3 months.

The increased bone ALP activity in hyperthyroidism can be explained by a direct action of thyroid hormones on osteoblasts (Sato et al, 1987). Osteocalcin was also raised in group 1 patients (Table 7.5) with a mean level approximately 2.4 times that found in plasmapheresis donors (Table 5.4). Other studies (Garrel et al, 1986; Martinez et al, 1986) found osteocalcin levels in hyperthyroidism which were 2 to 3 times those of control subjects. Both markers of osteoblast function (bone ALP and osteocalcin) returned to levels which were within or just above the reference range on treatment of hyperthyroidism (compare group 1 with group 2 - Tables 7.2 and 7.5).

There was no correlation between bone ALP activity or osteocalcin and free T_4 in group 1 patients. Martinez et al, 1986, found significant correlations for total ALP and osteocalcin with free T_4. Osteocalcin showed a significant correlation with bone ALP in both group 1 and group 2 patients. There has been only one study which has looked specifically at bone ALP, together with osteocalcin, in hyperthyroid patients (Duda et al, 1988). They found concordant results for the two markers.

Group 1 patients had higher mean concentrations of calcium than group 2 (Table 7.5) although nearly all values were within the reference range (Figure 7.2). Mean PTH levels were lower in group 1 compared to group 2 (Table 7.5). These results are expected since the physiological response to increases in circulating calcium concentrations is suppression of PTH release from the parathyroid glands. Thyroid hormones increase bone resorption in organ culture (Mundy et al, 1976). Thus, thyroid hormones are likely to be the cause of the increased plasma calcium in group 1. Other evidence for increased osteoclastic activity comes from the demonstration of increased urinary excretion of calcium, phosphate and hydroxyproline in hyperthyroidism (Mosekilde et al, 1978).

The pattern of higher intestinal ALP activities in BO secretors compared to A secretors and non-secretors was demonstrated in group 1, group 2 and in the controls (Table 7.3). Intestinal ALP activity was not different between group 1, group 2 and the controls when all three categories of blood group/secretor status category were considered together or when BO secretors were considered alone. The slightly higher intestinal ALP activities in A secretors and non-secretors of groups 1 and 2 compared with the control group may be due to non-specific interference by non-intestinal ALP in the ELISA.

In conclusion, total, liver and bone ALP were significantly higher in the hyperthyroid group compared to the euthyroid group or the blood donors. Bone ALP was more frequently and more markedly abnormal than liver ALP. There was no difference in intestinal ALP between the patient groups.

The abnormalities in liver and bone ALP are largely resolved on treatment of hyperthyroidism. Thus, in most cases, the finding of a raised total ALP in an untreated hyperthyroid patient would not seem to require further investigation, unless the abnormality persists after treatment.

CHAPTER 8

PLASMA ALKALINE PHOSPHATASE FORMS IN PATIENTS ON HAEMODIALYSIS

8.1. Introduction

Metabolic bone disease is a common occurrence in patients with chronic renal failure maintained on long term dialysis and can present clinically with symptoms such as bone pain and deformity. A number of factors may be involved. These factors include inadequate 1-hydroxylation of 25-hydroxycholecalciferol by the diseased kidneys, phosphate retention, secondary hyperparathyroidism and chronic acidosis. The picture may be further complicated by the osteomalacia that can result from aluminium toxicity (Feinfeld and Sherwood, 1988).

It is important to detect the presence of renal bone disease at an early stage, when treatment with vitamin D metabolites may prevent the progression of the disease. Only patients with evidence of bone abnormalities should receive treatment since over-treatment with vitamin D or its metabolites may cause excessive bone resorption. At present, skeletal X-rays and measurement of total ALP are often used routinely to detect bone disease but these become abnormal relatively late in the disease process. Bone histology would show early changes but this requires a bone biopsy which is invasive.

Of the ALP forms present in plasma, bone ALP is the one of interest in relation to patients with chronic renal failure. Therefore, this study was primarily concerned with bone ALP although intestinal, liver and, when present, high-molecular-mass ALP were also measured. Quantitative measurements of bone ALP activity were compared to osteocalcin and PTH concentrations. The relationship between these biochemical measurements and the presence, on X-rays, of bone erosions and vascular calcification was studied.

Total ALP activity is easy to measure and readily available in routine laboratories. Measurement of bone ALP activity, on the other hand, is time-consuming and requires

specialized techniques. Therefore, the possibility of using total ALP in association with biochemical indices of liver disease, to obtain a crude but useful assessment of bone ALP was investigated.

Intestinal ALP has been reported to be increased in patients with chronic renal failure (Walker, 1974; Skillen and Pierides, 1977). In the present study, intestinal ALP activities were measured and related to blood group and secretor status, as described in previous chapters. This was done to confirm reports of an increased intestinal ALP activity in patients with chronic renal failure. It was also done to see if the increase in intestinal ALP was sufficient to cause a rise in total ALP. This would then limit the diagnostic value of total ALP in the detection of bone disease.

8.2. Patients

Seventy five patients with end-stage chronic renal failure were studied. Details of these patients are given in Table 8.1. Thirty four patients were being treated by hospital haemodialysis and 41 by home haemodialysis. Seventeen of the patients had been treated by haemodialysis using softened water until 1979, since when dialysis in all patients had been carried out using reverse osmosis-treated water with regular monitoring of dialysis fluid and plasma aluminium concentration.

All patients were treated with Alucap in a dose of 1.4 - 2.8 g daily with the aim of maintaining plasma phosphate less than 2 mmol/L. Twelve patients were being treated with alfacalcidol in a dose of 0.25 - 0.5 μg daily. Nine patients had required parathyroidectomy in the past, 7 of these had been treated by total parathyroidectomy with forearm auto-transplantation.

Table 8.1. Details of the 75 patients studied.

Sex	Number	Mean age (range) years	Mean duration of dialysis (range) years
Male	48	49 (19-74)	6.7 (0.3-24)
Female	27	47 (18-68)	4.9 (0.4-16)

8.3. Samples

The following samples were collected on the pre-dialysis day:

1. Blood into a lithium heparin tube
2. Blood into a plain glass tube
3. Saliva (from 38 of the patients)

Plasma and serum were separated within 2 hours of collection and kept at -20°C. A portion of whole blood was kept at 4°C and the saliva sample kept at - 20°C.

8.4. Measurements

On plasma: total ALP; liver and bone ALP; intestinal ALP (by ELISA); osteocalcin (by the commercial radioimmunoassay kit from CIS UK Ltd and by the in-house radioimmunoassay); bilirubin, ALT and GGT; urea and creatinine; calcium, phosphate, magnesium and aluminium.

On serum: PTH and 25-hydroxyvitamin D (25-OH-D).

On whole blood: ABO and Lewis blood groups.

On saliva: secretor status.

8.5. Radiological assessment

The presence of erosions and vascular calcification on X-rays was assessed by Dr R.J. Winney, Medical Renal Unit, The Royal Infirmary of Edinburgh. A semi-quantitative score of 0 up to 3 was given which related to the severity and extent of erosions and calcification.

8.6. Results

Urea and creatinine

Mean plasma urea of the patients studied was 26.8 mmol/L (SD = 6.5 mmol/L, range 13.3 - 44.7 mmol/L); mean plasma creatinine was 1175 μmol/L (SD = 278 μmol/L, range 405 - 1970 μmol/L).

Measurements relating to bone disease

Mean levels in plasma of total ALP, bone ALP and osteocalcin and, in serum, of PTH are shown in Table 8.2. The table also shows the number of patients who had results above the reference range (Chapter 5). The correlation matrix between these variables and the presence of bone erosions is shown in Table 8.3. The data presented for osteocalcin, unless otherwise indicated, relate to measurements by the commercial kit from CIS UK Ltd.

Table 8.2. Total ALP, bone ALP, osteocalcin and PTH in patients on haemodialysis.

	Mean	SD	Range	% abnormal
Total ALP	114 U/L	61 U/L	47-367 U/L	37%
Bone ALP	61 U/L	58 U/L	11-349 U/L	33%
Osteocalcin	58 μg/L	48 μg/L	8-246 μg/L	99%
PTH	317 ng/L	566 ng/L	2-3870 ng/L	81%

Table 8.3. Correlation matrix for total ALP, bone ALP, osteocalcin, PTH and the presence of erosions

	Bone ALP	Osteo-calcin	PTH	Bone erosions
Total ALP	0.82**	0.60**	0.46**	0.43**
Bone ALP		0.78**	0.50**	0.56**
Osteocalcin			0.62**	0.59**
PTH				0.58**

**$p<0.001$

Total ALP was raised in 28 (37%) of the patients and was significantly correlated with osteocalcin and PTH. However, bone ALP was more closely correlated with osteocalcin than total ALP with osteocalcin (Table 8.3). In an attempt to improve the correlation between total ALP and osteocalcin, those patients who might have been expected to have a raised liver ALP, as predicted from a raised plasma GGT activity, were eliminated from the correlation. This resulted in an improvement in the correlation between total ALP and osteocalcin, the correlation coefficient rising from 0.60 to 0.73. This is demonstrated in Figure 8.1 in which patients with raised plasma GGT activity can be seen to cluster in an area of high total ALP activity and low osteocalcin concentration.

Bone ALP was raised in 25 (33%) of the patients and was closely correlated with osteocalcin (Figure 8.2) and, to a lesser extent, with PTH (Table 8.3). Seven patients with a total ALP activity within the reference range had a raised bone ALP activity. Although osteocalcin correlated well with bone ALP, a much higher percentage (99%) of the patients had raised osteocalcin levels (Table 8.2). Because of this finding of a higher percentage of abnormal osteocalcin concentrations compared to bone ALP activities, the correlation between bone ALP and osteocalcin in patients with renal failure was compared to the correlation found earlier in hyperthyroid patients (Chapter 7). As discussed previously, hyperthyroid patients have increased bone turnover but usually have normal renal function. Figure 8.3 shows that, for a given activity of bone ALP, renal failure patients have higher plasma osteocalcin levels than hyperthyroid patients.

Table 8.4 shows the mean (SD and range) for calcium, phosphate, magnesium and aluminium in plasma and 25-OH-D in serum. These variables did not show any statistically significant correlations with other biochemical measurements or radiological changes of bone disease. The plasma aluminium concentration was strongly correlated only with the duration of dialysis ($r = 0.47$; $p<0.001$). Although 25-OH-D was low in 36% of patients, there was no significant relation to any of the other indices measured.

Table 8.4. Calcium, phosphate, magnesium, aluminium and 25-OH-D in patients on haemodialysis.

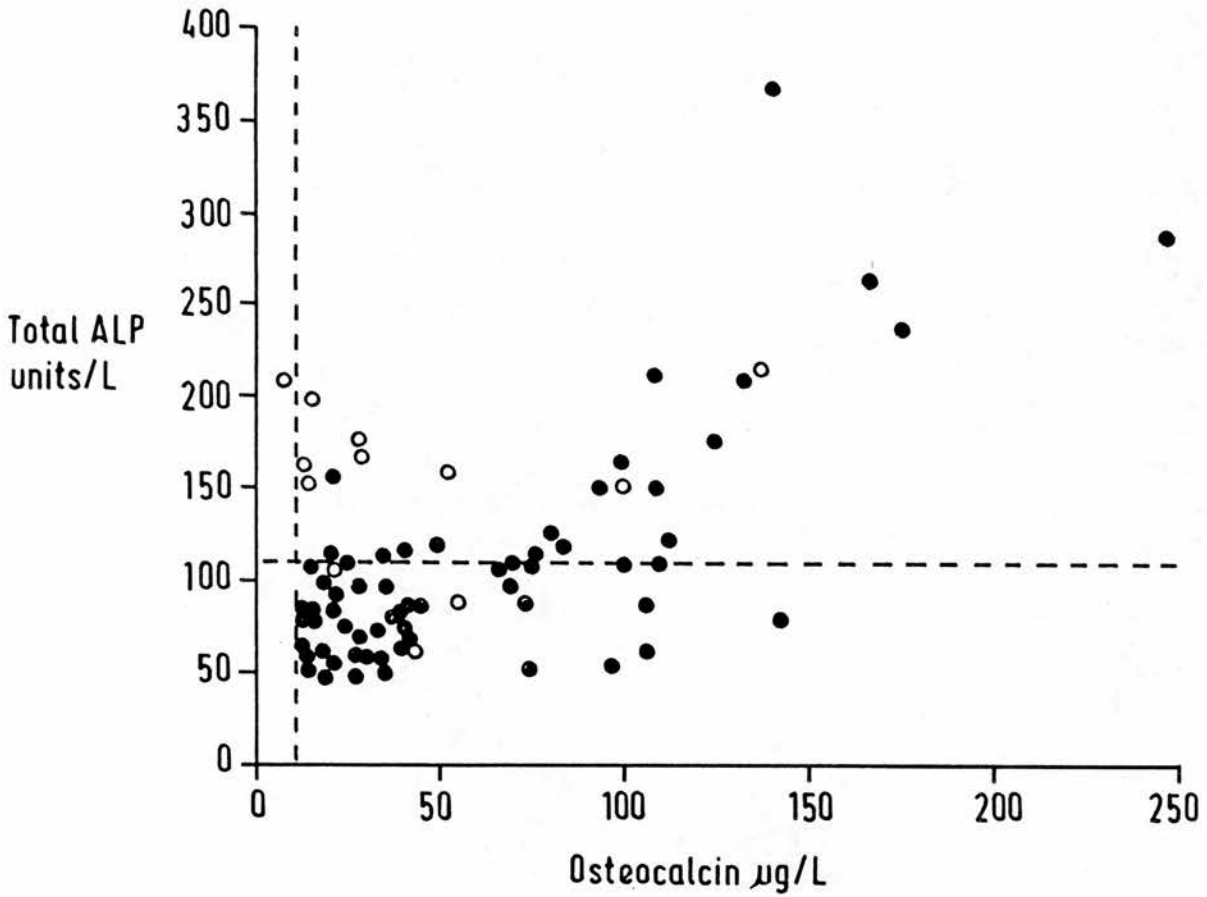
	Mean	SD	Range
Calcium (mmol/L)	2.45	0.20	1.66-2.84
Phosphate (mmol/L)	2.00	0.60	0.71-3.70
Magnesium (mmol/L)	1.32	0.20	0.87-1.73
Aluminium (μmol/L)	3.03	2.03	0.60-13.5
25-OH-D (μg/L)	15.2	8.7	4.2-35.7

Bone erosions were visible, on X-ray, in 11 (15%) of the patients. All had raised osteocalcin and PTH levels. Two patients with grade 1 erosions had total and bone ALP activities within the reference range; four patients had normal total ALP with a raised bone ALP activity and one patient had a raised total ALP (caused by an increased liver ALP) with normal bone ALP activity. All other patients with erosions had a raised total and bone ALP activity.

There were significant correlations between bone ALP, osteocalcin, PTH and the presence of bone erosions (Table 8.3). Patients with erosions had higher values for the various biochemical indices of bone metabolism than other patients (9/11 in the upper quartile of the osteocalcin distribution, 7/11 and 6/11, respectively, in the upper quartile of the PTH and bone ALP distribution). There was no correlation between duration of dialysis and presence of erosions.

Vascular calcification was present in 49 patients and was widespread and severe in 16 patients. There was a strong correlation with age ($r = 0.70$) and a significant negative correlation with osteocalcin ($r = -0.30$). There was no clear relationship between vascular calcification and erosions, only one patient with grade 1 erosions having severe vascular calcification.

Figure 8.1. Correlation between plasma total ALP activity and osteocalcin. Open circles represent patients with raised plasma GGT activity (over 55 U/L in males or 35 U/L in females), closed circles represent patients with normal GGT activity.



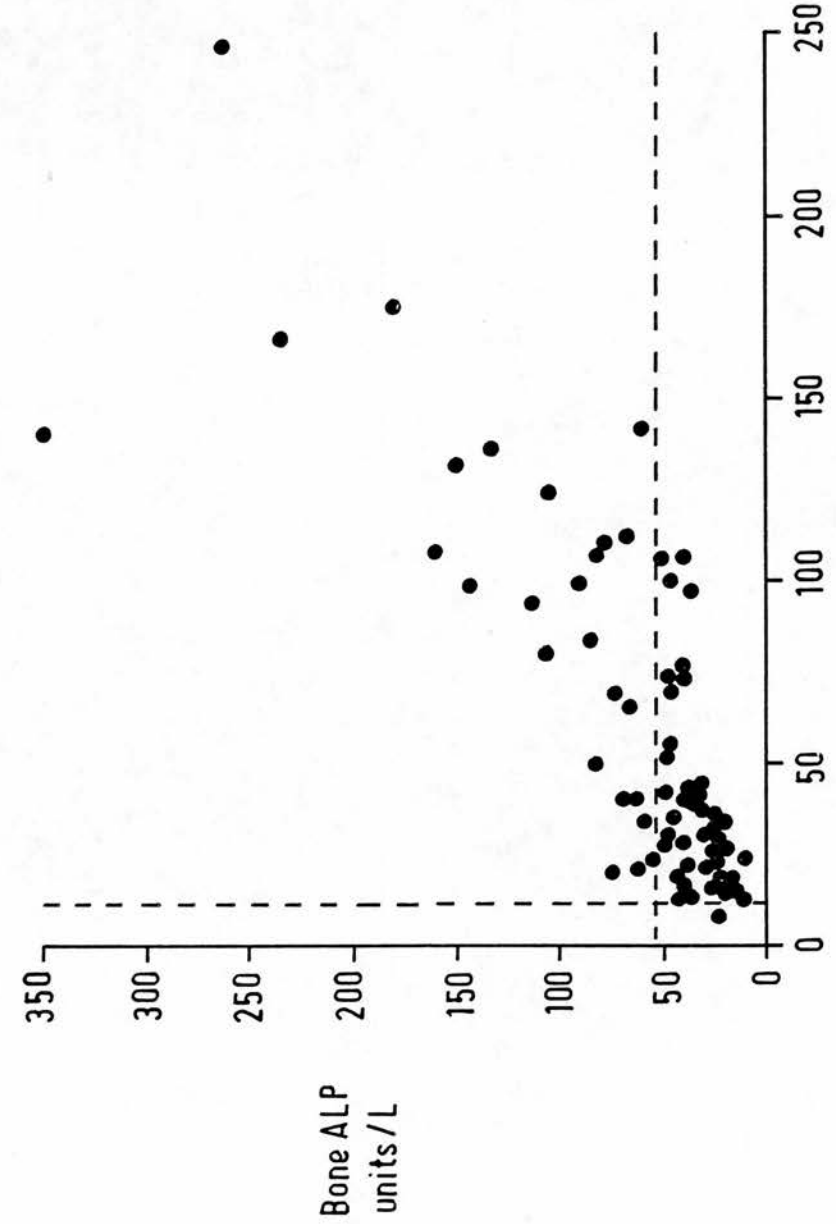
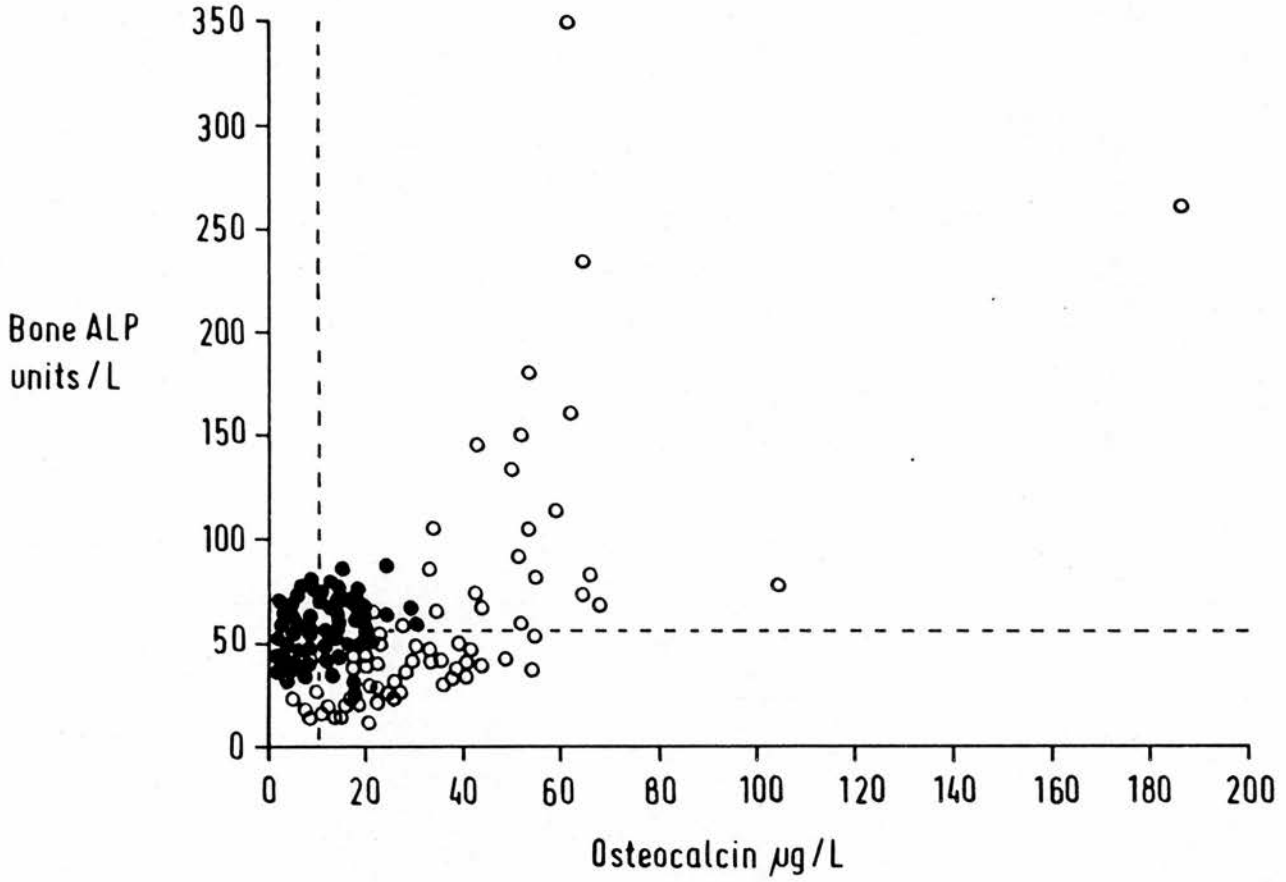


Figure 8.2. Correlation between plasma bone ALP activity and osteocalcin.

Figure 8.3. Correlation between plasma bone ALP activity and osteocalcin for patients with renal failure (open circles) and for hyperthyroid patients (closed circles). Osteocalcin was measured by the in-house radioimmunoassay.



Measurements relating to liver disease.

Only 16 (21%) of the patients had any biochemical evidence of liver abnormality. Twelve (16%) of these patients had a raised liver ALP activity (Table 8.5) and in 10 of them the total ALP and GGT activities were also raised. In 5 of the patients with raised liver ALP, there was also staining for high-molecular-mass ALP at the origin. Mean high-molecular-mass ALP in these latter patients was 22 U/L (range 6 - 60 U/L). Abnormalities of these measurements and of bilirubin and ALT are summarised in Table 8.5.

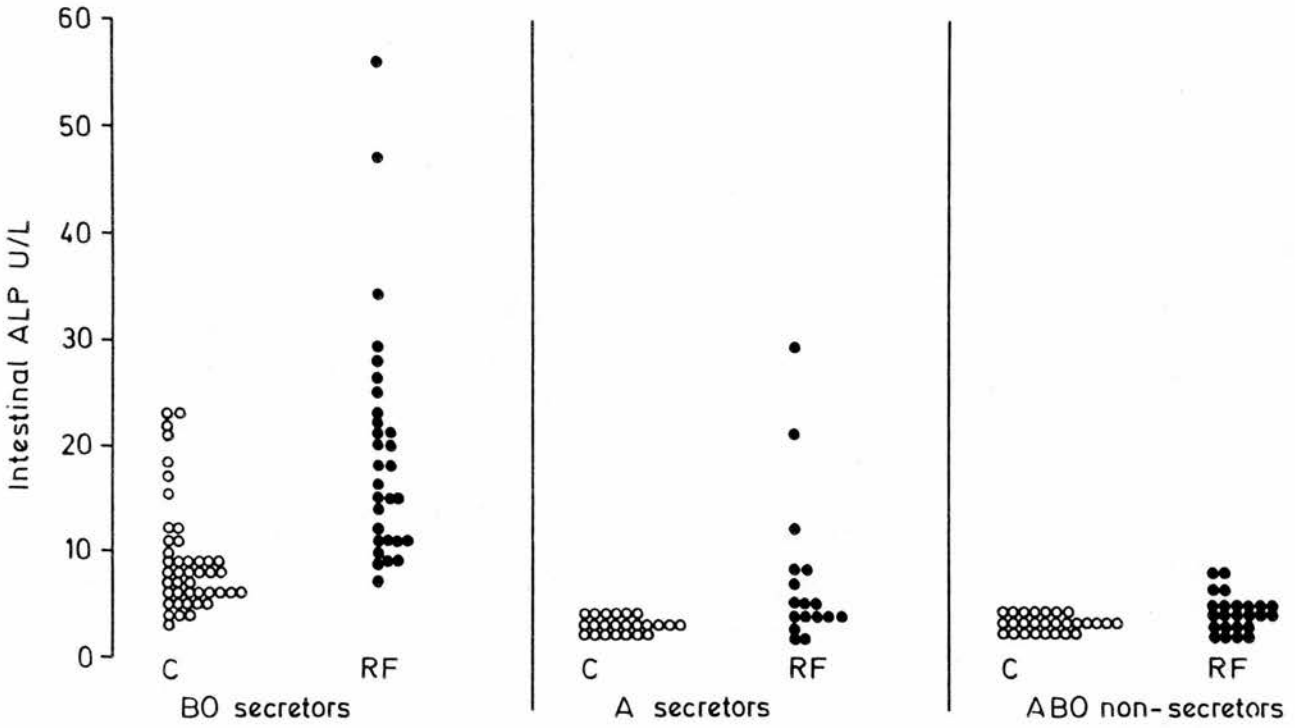
Table 8.5. Number (percentage) of abnormal results for bilirubin, ALT, GGT, liver and high-molecular-mass ALP.

	Number (%)
Bilirubin	2 (3)
ALT	5 (7)
GGT	12 (16)
Liver ALP	12 (16)
High-molecular-mass ALP	5 (7)

Intestinal ALP, blood group and secretor status

Figure 8.4 shows the activities of intestinal ALP in patients with renal failure. These were compared with intestinal ALP activities in blood donors (Section 5.1: control group 2). Within each of the categories of blood group and secretor status, renal failure patients had significantly higher intestinal ALP activities compared to the control group ($p<0.001$ in all cases). Renal failure patients who were BO secretors had higher intestinal ALP activities than renal failure patients who were A secretors or ABO non-secretors ($p<0.01$). Patients who were A secretors did not have significantly different intestinal ALP activities than non-secretors, although there were 2, possibly 3, patients who had activities which stood out as being considerably higher than the rest of the A secretor or ABO non-secretor group (Figure 8.4).

Figure 8.4. Plasma intestinal ALP activity in the control group (open circles) and renal failure patients (closed circles) for the three blood group/secretor status categories (BO secretors, A secretors and ABO non-secretors).



Eight patients had intestinal ALP activities greater than 23 U/L (8 BO secretors and one A secretor) and in these patients intestinal ALP activities accounted for a significant proportion of total ALP activity (mean percentage intestinal ALP activity 30%, range 16 - 42%). Three of these patients had a raised total ALP. In one of these patients, the raised intestinal ALP was the only reason for the increase in total ALP activity above the reference range. In the other patients, intestinal ALP, together with bone ALP, was responsible for the raised total ALP. None of the eight patients had abnormal plasma liver ALP or GGT activities.

8.7. Discussion

Renal bone disease is, at present, detected by monitoring skeletal X-rays and measuring plasma total ALP. These are both insensitive and, in the case of total ALP, non-specific. The present study was mainly concerned with the role of total ALP, bone ALP and other bone-related biochemical indices in the detection of renal bone disease. Biochemical indices were related to radiological changes.

Approximately 20% of patients in this study had biochemical evidence of disturbed liver function. Disturbed liver function, resulting in a raised liver ALP, will limit the diagnostic value of total ALP in bone disease. This is demonstrated by the poorer correlations obtained for total ALP with osteocalcin and bone erosions, when compared to bone ALP (Table 8.3). This problem can be overcome by measurement of plasma GGT together with total ALP. When patients with a raised GGT activity were eliminated from the correlation between total ALP and osteocalcin, the correlation coefficient obtained ($r = 0.73$) was similar to that obtained for bone ALP with osteocalcin ($r = 0.78$).

Specific measurements of the bone form of ALP are therefore useful in patients with disturbed liver function in whom bone disease is suspected. There were also seven patients with a total ALP activity within the reference range with a raised bone ALP activity which suggests that bone ALP, in addition to its greater specificity, may be more sensitive than total ALP. Bone histology was not available in this study; it is therefore not possible to say whether these patients with a raised bone ALP had

histological evidence of renal bone disease, although four of the seven patients had evidence of bone erosions on X-rays.

Bone ALP activity paralleled osteocalcin quite closely (Figure 8.2) which is to be expected since both measurements reflect osteoblastic activity. Both bone ALP and osteocalcin were positively correlated with the presence of bone erosions (Table 8.3). The major difference between the two measurements was that whereas bone ALP was abnormal in one third of the patients, osteocalcin was abnormal in nearly all of them (Table 8.2). Other workers have also found osteocalcin to be abnormal in virtually all patients with chronic renal failure and found bone ALP to be increased in approximately 35% of patients (Duda et al, 1988).

Osteocalcin levels tend to rise sharply in patients with renal failure when the GFR is less than 20 ml/min (Delmas et al, 1983). Therefore, the raised osteocalcin levels in the present study are probably due to failure to excrete osteocalcin rather than to osteocalcin being a more sensitive index of bone turnover than bone ALP - which is not normally excreted in the urine. This conclusion is strengthened by data from patients with hyperthyroidism (Figure 8.3). This figure shows that, for a given bone ALP, patients with renal failure tend to have a higher plasma osteocalcin than patients with hyperthyroidism. On the other hand, Malluche et al, 1984, found that osteocalcin levels in thirty patients with renal osteodystrophy, in all of whom bone biopsy was performed, correlated better with histological findings than total ALP. They did not measure bone ALP in their study.

Parathyroid hormone stimulates osteoclastic bone resorption which in turn increases bone formation. The increase in bone formation is probably mediated by the generation of local coupling factors during the resorption process, although it is possible that PTH by itself, in low doses, increases bone formation (Mundy, 1987). It is therefore not surprising that PTH correlated with markers of osteoblast activity in this study (Table 8.3). Serum PTH also correlated with bone erosions but was no better in predicting the presence of erosions than bone ALP or osteocalcin.

There was marked variation in parameters of bone metabolism at any given level of PTH. In particular, 36 patients had raised PTH levels with normal bone ALP activity. This may reflect the limited sensitivity of bone ALP in detecting disturbed bone

metabolism or it may be due to the presence of some factor such as aluminium which is known to inhibit bone formation. However, in this study plasma aluminium did not correlate with PTH or any of the other biochemical indices of bone disease measured.

There seems to be no particular advantage in the measurement of PTH over bone ALP or osteocalcin in the assessment of bone disease. In fact, it seems more logical to use a product of bone activity to assess bone disease rather than a hormone, the level of which may be affected by factors such as plasma concentrations of calcium, phosphate or magnesium.

Plasma levels of calcium, phosphate, magnesium, aluminium and 25-OH-D did not correlate with bone ALP, osteocalcin, PTH or radiological findings. In the case of phosphate, magnesium and aluminium, mean levels in plasma in patients were above the reference range (Table 8.4). Levels of 25-OH-D, which were used in this study mainly to assess the nutritional status of patients, were found to be below the optimal concentration in one third of the patients.

Vascular calcification showed a positive correlation with age and a negative correlation with osteocalcin, which was itself negatively correlated with age. Therefore, the relationship between vascular calcification and osteocalcin may be a result of the relationship of both of these parameters with age.

In contrast to a previous report (Alpers et al, 1988), the results of the present study have shown that a raised intestinal ALP very rarely interferes with the interpretation of total ALP when assessing bone disease in patients with chronic renal failure. Intestinal ALP was, however, significantly higher in renal failure patients than in controls when the group was considered as a whole or subdivided into blood group/secretor status categories (Figure 8.4). As was found previously in healthy adults and in diabetic and hyperthyroid patients (Chapters 5, 6 and 7), the results from BO secretors were higher than A secretors or non-secretors.

The reason for the raised intestinal ALP activities, up to 42% of total ALP activity, in some patients with chronic renal failure is unclear. None of the patients with raised intestinal ALP activity showed any biochemical evidence of liver disease. It is therefore unlikely that the raised intestinal ALP activities in these patients were due to an inability

of the liver to clear intestinal ALP from the circulation, as has been described in patients with liver disease (Stolbach et al, 1967). Stepan et al, 1984, showed an inverse relationship between intestinal ALP and serum calcium and have suggested that serum calcium concentration affects the hepatic binding of intestinal ALP. However, there was no relationship found between intestinal ALP activity and plasma calcium concentrations in patients described in the present study. It has also been suggested (Alpers et al, 1988) that the kidney itself is the source of intestinal ALP, since kidneys removed during transplantation or at post-mortem were found to contain an intestinal-type alkaline phosphatase.

In conclusion, the results of this study suggest that, in the routine monitoring of patients with chronic renal failure for bone disease, measurements of plasma total ALP and GGT activity are sufficient in most cases. Plasma intestinal ALP activity, although often raised, rarely affects the interpretation of total ALP activity. Bone ALP measurement is useful in patients with liver disease in whom bone disease is suspected. There is some evidence that bone ALP is more sensitive than total ALP in detecting bone disease but this needs to be confirmed by further studies in which bone histology is performed. Bone histology would also be useful for defining an appropriate reference range for osteocalcin in patients with chronic renal failure.

CHAPTER 9

PLASMA ALKALINE PHOSPHATASE FORMS IN OBSTRUCTIVE LIVER DISEASE.

9.1. Introduction

Total ALP activities in plasma are very high in patients with obstructive liver disease, mainly because of a raised liver ALP activity. In addition, intestinal ALP can also be raised in the plasma of patients with liver disease, but the increase appears to be limited to patients with intrahepatic disease, particularly cirrhosis (Stolbach et al, 1967). The main factor responsible for the increased activities of intestinal ALP in liver disease is thought to be reduced clearance of the enzyme by the damaged hepatocyte (Warnes et al, 1981).

It has been suggested that the measurement of intestinal ALP activity may be of value in distinguishing between intra- and extrahepatic obstruction (Warnes et al, 1977). However, Collins et al, 1987, found that intestinal ALP activity had a poor sensitivity and specificity in the differential diagnosis of jaundice, even when the blood group of patients was taken into account.

As was described in previous chapters of this thesis, intestinal ALP activity in health and disease varies not only with blood group but also with the secretor status of the individual. In their study, Collins et al, 1987, did not take secretor status of patients into account when assessing the value of intestinal ALP in the differential diagnosis of obstructive liver disease. In addition, they measured intestinal ALP by the bromotetramisole method. This method was shown earlier (Section 2.5) to give falsely elevated intestinal ALP activities in some plasma samples. It is also important to mention that placental ALP, which may be present in patients with cholestasis (Domar et al, 1988) will interfere in the bromotetramisole method giving falsely elevated intestinal ALP activities.

The present study of patients with liver disease was carried out mainly to assess the value of intestinal ALP activity, measured by a specific immunological method (ELISA), in the differential diagnosis of obstructive liver disease. Results obtained using the ELISA method were compared to those obtained using bromotetramisole-inhibition. Intestinal ALP activity was related to both blood group and secretor status of the patients. High-molecular-mass ALP is raised in most patients with obstructive liver disease (Siede and Seiffert, 1983). Therefore, high-molecular-mass ALP was measured in the same series of patients as intestinal ALP to see if it was helpful in the differentiation of intra- from extrahepatic obstruction.

9.2. Patients

Forty three patients with intrahepatic disease and 26 patients with extrahepatic disease were studied (Table 9.1). Diagnoses in the two groups are given in Table 9.2 and were based on clinical examination, biochemical and serological tests and in some cases on liver biopsies and radiological investigations (endoscopic retrograde cholangiopancreatography: ERCP).

Table 9.1. Details of 69 patients studied.

	Intrahepatic	Extrahepatic
Number		
Total	43	26
Male	24	17
Female	19	9
Age (years)		
Mean	56	66
SD	11	16
Range	27-76	27-86

Table 9.2. Diagnoses (number in each diagnostic group) of patients with intra- or extrahepatic disease.

Intrahepatic	Extrahepatic
Cirrhosis(13)	Carcinoma:
Hepatitis(11)	bile ducts(5)
Liver failure(4)	head of pancreas(7)
Hepatoma(3)	porta hepatis(1)
Primary biliary cirrhosis(2)	Gallstones/cholecystitis(6)
Ascites(2)	Pancreatitis(3)
Haemochromatosis(2)	Pancreatic pseudocyst(1)
Liver metastases(2)	Apudoma in pancreas(1)
Alcoholic fatty liver(1)	Stricture in bile duct(1)
Caroli's syndrome(1)	Choledochal cyst(1)
Liver abscess(1)	
Sclerosing cholangitis(1)	

9.3. Samples

A lithium heparin blood sample and saliva sample (available from 17 patients) were collected. A portion of whole blood was kept at 4°C. The saliva samples and the separated plasma were kept at -20°C until analysis.

9.4. Measurements

On plasma: total ALP; liver ALP; intestinal ALP (by ELISA and by the bromotetramisole method); high-molecular-mass ALP (by ion-exchange).
On whole blood: ABO and Lewis groups.
On saliva: secretor status.

9.5. Results

Intestinal ALP, blood group and secretor status.

Intestinal ALP activity was related to the three blood group/secretor status categories as described earlier in this thesis. As was found previously in blood donors (Chapter 5) and in diabetic and hyperthyroid patients (Chapters 6 and 7), intestinal ALP activities of A secretors were similar to activities in non-secretors. Therefore, because the number of patients in the A secretor and non-secretor categories were small, these two categories were combined together as a single category (non-secretors plus group A) for the two patient groups (intrahepatic and extrahepatic obstruction).

Patients with intrahepatic obstruction had significantly higher intestinal ALP activities (measured by ELISA) than patients with extrahepatic obstruction when all categories were considered together or when BO secretors were considered alone (Table 9.3). There was no statistical difference in intestinal ALP activities of patients with intra- and extrahepatic obstruction for the non-secretor plus group A category, although mean intestinal ALP was higher in patients with intrahepatic obstruction.

In contrast, when intestinal ALP activity was measured by the bromotetramisole method, only the BO secretor category of the intrahepatic group had significantly higher mean levels compared to the extrahepatic group (Table 9.3). This difference between the bromotetramisole and ELISA methods is illustrated in Figure 9.1, where it can be seen that the ELISA method differentiates more clearly between intra- and extrahepatic obstruction than the bromotetramisole method for both blood group/secretor status categories.

Placental ALP, which has been reported to be present in patients with liver disease (Domar et al, 1988), would be measured as "intestinal ALP" by the bromotetramisole method. To check for the presence of placental ALP, plasma samples from all patients in this study were heated at 65^{o}C for 10 minutes. The residual ALP activities were less than 1 U/L in all cases, indicating that placental ALP was not present in significant enough quantities to account for the higher intestinal ALP activities measured by the bromotetramisole method.

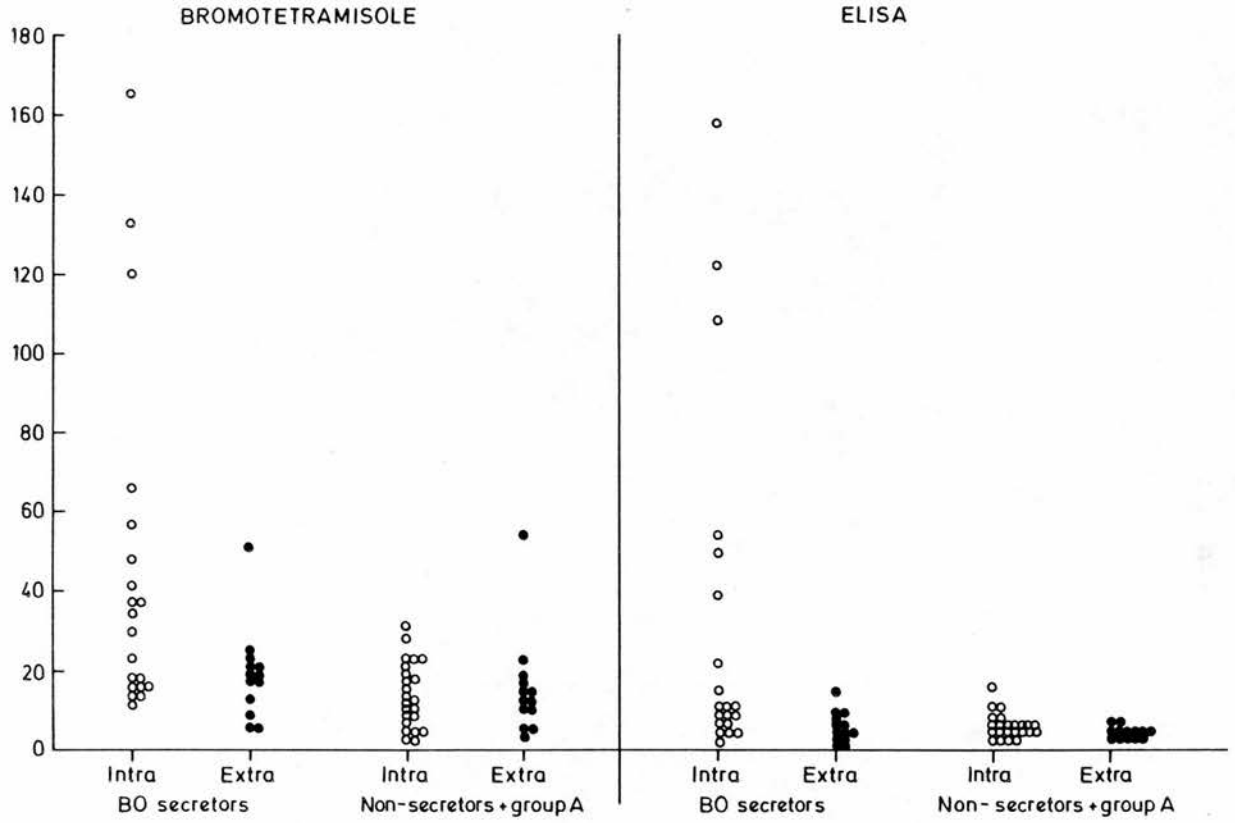
Table 9.3. Plasma intestinal ALP activities, measured by ELISA or by the bromotetramisole method, in patients with intrahepatic and extrahepatic obstruction.

		Intestinal ALP activity (U/L)	
		ELISA	Bromotetramisole
	n	Mean (SD)	Mean (SD)
Intrahepatic			
All	43	18.3*(33.2)	28.8 (34.0)
BO secretors	20	32.8*(44.9)	45.8*(43.7)
Non-secretors plus group A	23	5.8 (3.0)	14.1 (8.2)
Extrahepatic			
All	26	5.5 (2.5)	17.5 (12.0)
BO secretors	14	6.1 (3.3)	18.1 (11.6)
Non-secretors plus group A	12	4.7 (1.0)	16.7 (12.9)

Statistical tests (Mann-Whitney) were done comparing intestinal ALP activities (measured by ELISA or by the bromotetramisole method) in patients with intrahepatic obstruction to those with extrahepatic obstruction, for all patients together or divided into blood group/secretor status categories (BO secretors, non-secretors plus group A). Asterisk indicates level of significance (*$p<0.05$).

Values for intestinal ALP, measured by the ELISA method, which differentiated most clearly between intra- and extrahepatic obstruction were chosen. These were an intestinal ALP activity of 20 U/L for patients in the BO secretor category and 7 U/L in the non-secretor plus group A category. The sensitivity and specificity of intestinal ALP activity measurement for intrahepatic obstruction were 23% and 100% respectively (Table 9.4). The predictive value of a positive result (intestinal ALP activity greater than 20 U/L for BO secretors and greater than 7 U/L for non-secretors plus group A) in the diagnosis of intrahepatic obstruction was 100%. The predictive value of a negative

Figure 9.1. Plasma intestinal ALP activities, measured by the bromotramisole method or by ELISA, in patients with intrahepatic obstruction (open circles) and patients with extrahepatic obstruction (closed circles) for the two blood group/secretor status categories.



result (intestinal ALP activity less than or equal to 20 U/L for BO secretors and less than or equal to 7 U/L for non-secretors plus group A) in excluding a diagnosis of intrahepatic obstruction was 44% (Table 9.4).

Table 9.4. Diagnostic value of plasma intestinal ALP activity in the detection of intrahepatic obstruction.

	Number (%)
Sensitivity[a]	10/43 (23)
Specificity[b]	26/26 (100)
Predictive value of a positive result[c]	10/10 (100)
Predictive value of a negative result[d]	26/59 (44)

Proportion of patients [a] with intrahepatic obstruction giving a positive result, [b] without intrahepatic obstruction giving a negative result, [c] with positive result who had intrahepatic obstruction and [d] with a negative result who did not have intrahepatic obstruction.
Positive result: greater than 20 U/L for BO secretors and 7 U/L for non-secretors plus group A; negative result: less than or equal to 20 U/L for BO secretors and 7 U/L for non-secretors plus group A.

Total, liver and high-molecular-mass ALP.
Plasma total and liver ALP activities were significantly higher in patients with extrahepatic obstruction compared to patients with intrahepatic obstruction (Table 9.5). There was no significant difference in high-molecular-mass ALP activity between the two groups although the mean activity was higher in patients with extrahepatic obstruction (Table 9.5).

Table 9.5. Plasma total, liver and high-molecular-mass (HMM) ALP activities in patients with intra- or extrahepatic obstruction

	ALP activity (U/L)	
	Intrahepatic	Extrahepatic
	Mean (SD)	Mean (SD)
Total ALP	258 (219)	558* (513)
Liver ALP	157 (194)	432* (419)
HMM-ALP	39 (62)	79 (114)

Statistical tests were carried out comparing activities in patients with intrahepatic obstruction with those in patients with extrahepatic obstruction. Asterisk indicates level of significance (* $p<0.05$).

9.6. Discussion

Intestinal ALP enters the general circulation via the intestinal lymphatics and thoracic duct and is cleared from the circulation by a galactose-specific receptor on the hepatocyte (Moss, 1989). Several workers have reported an increase in plasma intestinal ALP activity in liver disease particularly cirrhosis (Fishman et al, 1965; Stolbach et al, 1967; Barakat et al, 1971). In cirrhosis, there is a reduction in the number of functional hepatocytes resulting in a reduced clearance of the enzyme and a rise in its activity in plasma. On the other hand, in extrahepatic obstruction, bile salts may be absent from the intestinal lumen causing reduced solubilisation of ALP from the enterocyte and reduced entry of intestinal ALP into the circulation. It is on this basis that measurement of intestinal ALP in plasma has been put forward as a test for distinguishing intra- from extrahepatic obstruction (Warnes et al, 1977). However, in a later study by Collins et al, 1987, the test was found to lack sensitivity for the diagnosis of intrahepatic obstruction. It was also found not to be specific for intrahepatic disease.

In the present study, intestinal ALP was measured by ELISA and cut-offs were determined which distinguished intra- from extrahepatic obstruction for each of the blood group/secretor status categories. The sensitivity of intestinal ALP for intrahepatic obstruction was found to be poor (23%); however the test had a specificity of 100% with a predictive value of a positive test of 100% (Table 9.4). Thus, a patient with liver disease is likely to have an intrahepatic lesion if the plasma intestinal ALP activity is greater than 20 U/L (BO secretor category) or 7 U/L (non-secretor plus group A category).

Collins et al, 1987, also found that intestinal ALP showed poor sensitivity (32%) in the diagnosis of intrahepatic obstruction. However, unlike the present study, they found that the test, in addition to its poor sensitivity had a specificity (86%) which was not absolute. They related intestinal ALP activity to blood group of patients but did not take secretor status into account which may explain the poor specificity found in their study. They measured intestinal ALP by bromotetramisole-inhibition. This method was found, in the present study, to differentiate poorly between intra- and extrahepatic obstruction especially for the non-secretor plus group A category (Figure 9.1).

There was a large overlap in high-molecular-mass ALP activities between the two groups and measurement of this form of ALP was not of value in distinguishing between intra- and extrahepatic obstruction. Similar results were found by Wenham et al, 1985. Both total and liver ALP were higher in the extrahepatic patient group (Table 9.5).

In conclusion, the measurement of plasma intestinal ALP activity by the ELISA method, although not sensitive, is specific for intrahepatic obstruction. Its measurement, together with radiological investigations, may be of value in determining the cause of obstruction in a patient presenting with liver disease.

CHAPTER 10

CONCLUSIONS

The aims of this thesis were to select reliable methods for the quantitation of the main ALP forms in plasma and to assess the value of these measurements in specific disease groups. This concluding chapter will consider:

(1) The methods chosen for the measurement of each ALP form, with emphasis on the suitability of the method for use in a routine diagnostic laboratory.
(2) The value of the measurement of ALP forms in the management of patients in relation to the clinical studies carried out and the contribution of these studies to the understanding of disease mechanisms.

10.1. Choice of methods

Polyacrylamide gel electrophoresis, with densitometric scanning of the bands, was the method selected for the measurement of liver and bone ALP. Partial treatment with neuraminidase, prior to electrophoresis, was used to enhance the separation between the liver and bone forms. The selected method showed satisfactory precision, correlated well with the heat-inactivation and lectin-precipitation methods and was not affected by the presence of either intestinal or high-molecular-mass ALP. Moreover, a raised activity of one ALP form (liver or bone) did not interfere in the measurement of the other ALP form.

The modified electrophoretic method used in this thesis for the quantitation of the liver and bone forms was specific and reliable; it was, however, complex and time-consuming to carry out. These are serious disadvantages, especially when considering the use of this method for the measurement of liver and bone ALP in large numbers of samples in a routine laboratory setting. This problem will be resolved only when monoclonal antibodies reacting specifically with the liver and bone forms become available. In the meantime, it may be possible to use measurements of

total ALP, GGT and osteocalcin (all of which are relatively easy to carry out) to define the liver and/or bone source of a raised total ALP in the majority of samples, reserving quantitation of liver and bone ALP for the more difficult cases.

The ELISA method was selected for the measurement of intestinal ALP because of its greater specificity compared with bromotetramisole-inhibition. The ELISA method gave intestinal ALP activities which were clinically of more value than those obtained by bromotetramisole-inhibition (Chapter 9). However, the bromotetramisole method has some advantages over the ELISA method: it is easier to perform, as well as more precise and it involves the use of readily available reagents. The bromotetramisole method is therefore more suited to a routine laboratory setting where it can provide a rapid means of quantitation. In all cases, electrophoresis should be used to confirm the presence of intestinal ALP in the sample.

Ion-exchange chromatography was the method selected for the measurement of high-molecular-mass ALP. This method was found to be more precise than the immunoprecipitation method, although it was labour-intensive. When compared to the ion-exchange method, immunoprecipitation gave results which were similar for most samples. The immunoprecipitation method is easy to perform and is more suited for use in a routine laboratory.

10.2. Value of the measurement of individual alkaline phosphatase forms

a. Clinical studies

The source of a raised total ALP in the plasma of patients with diabetes mellitus and with hyperthyroidism was established by individual quantitation of ALP forms. In the majority of patients, the abnormality was found to be due to the primary disease alone (diabetes or hyperthyroidism) and did not indicate an additional pathology. The raised intestinal ALP in diabetics may be due to an abnormal response in these patients to food ingestion. It is less likely to be the result of intestinal disease or of liver disease. Quantitation of individual ALP forms will still be required to identify the small number of diabetics with a raised plasma total ALP (in a fasting sample), which is not the result of a raised intestinal ALP. In hyperthyroidism, the abnormalities relating

to liver and bone ALP were shown to be a consequence of the thyrotoxic state and were not present, to any great extent, in euthyroid patients. Quantitation of ALP forms in hyperthyroid patients is important in those patients where the abnormality in total ALP persists after successful treatment of thyrotoxicosis.

This thesis has demonstrated the importance of measurements of individual forms of ALP in plasma in certain clinical settings. Thus, while in the majority of patients with chronic renal failure, the measurement of total ALP together with GGT was sufficient for the investigation and subsequent monitoring of bone disease, specific measurement of bone ALP was necessary in those patients with co-existent liver disease. There was evidence to suggest that bone ALP was more sensitive than total ALP in the detection of renal bone disease. Intestinal ALP, measured by a specific immunological method, was of value in the differential diagnosis of obstructive liver disease, particularly in patients belonging to the BO secretor category. In contrast, measurements of total ALP, liver ALP and high-molecular-mass ALP differentiated poorly between intra- and extrahepatic obstruction.

b. Disease aetiology

Liver and high-molecular-mass alkaline phosphatase.
Very high activities of both liver and high-molecular-mass ALP were present in plasma from patients with obstructive liver disease, particularly in patients with extrahepatic obstruction (Chapter 9). It may well be that an extrahepatic obstruction is a more powerful inducer of liver ALP synthesis compared to an intrahepatic lesion. In addition, bile salts which tend to be present at higher concentrations in extrahepatic obstruction, will solubilise a greater proportion of membrane-bound ALP, giving rise to higher activities of high-molecular-mass ALP in plasma.

Minor abnormalities in liver ALP were present in diabetes mellitus; these were found mainly in patients with long-standing disease. In thyrotoxicosis, there was evidence of both hepatocellular damage and cholestasis, with increased activities in plasma of cytosolic enzymes (ALT and GST) and membrane-bound enzymes (liver ALP and GGT). The biochemical abnormalities found in a few patients with chronic renal failure

(elevations in liver ALP, high-molecular-mass ALP and GGT activity) indicate the presence of predominantly "cholestatic" liver disease although the cause is uncertain.

Bone alkaline phosphatase

An increase in plasma bone ALP activity was found in 62% of patients with hyperthyroidism and in 33% of patients with chronic renal failure. In chronic renal failure, the increased osteoblastic activity is a result, directly or indirectly, of raised circulating levels of PTH. However, in hyperthyroidism, where PTH levels are suppressed, thyroid hormones are responsible for the increased osteoblastic activity.

Bone ALP and osteocalcin were raised in roughly the same percentage of patients with hyperthyroidism (62% and 58% respectively). In individual patients, however, there were disparate results, so that some patients had a normal osteocalcin with a raised bone ALP, while others had a raised osteocalcin with a normal bone ALP (Figure 8.3). These results suggest that the synthesis and release of bone ALP and osteocalcin occur at different times in the bone remodelling cycle in response to thyroid hormones. Hasling et al, 1987, have shown that the administration of exogenous tri-iodothyronine (T_3) to healthy volunteers causes a rise in serum osteocalcin in the first week with levels rising continuously up to 8 weeks, while serum total ALP increased 6 to 8 weeks after T_3 administration.

Plasma osteocalcin was raised in the majority of patients with chronic renal failure (99% of patients when osteocalcin was measured by the commercial kit from CIS UK Ltd; 89% of patients when osteocalcin was measured by the in-house radioimmunoassay). A much lower percentage of patients had a raised bone ALP (33%). This difference between bone ALP and osteocalcin is due mainly to reduced clearance of osteocalcin by the kidney in these patients. Local factors, such as tissue aluminium concentration, may also be relevant. Bone ALP and osteocalcin have different roles in bone remodelling: bone ALP is probably involved in mineralization, while osteocalcin may play a part in osteoclastic bone resorption (Lian et al, 1986). Thus, suppression of mineralization by aluminium may have a greater effect on bone ALP than on osteocalcin.

Both bone ALP activity and osteocalcin concentration were within the reference range in patients with diabetes mellitus indicating that the osteopenia, which can be present

in these patients, is not associated with increased osteoblastic activity. In fact, in diabetes, bone formation is reduced (Pietschmann et al, 1988).

Intestinal alkaline phosphatase.

Throughout this thesis, quantitative intestinal ALP measurements in plasma were related to blood group and secretor status. A pattern of higher intestinal ALP activities in the plasma of BO secretors compared to A secretors and non-secretors was found in blood donors and in patients with diabetes, hyperthyroidism and obstructive liver disease. A few patients with chronic renal failure belonging to the A secretor category formed an exception to the rule (Figure 8.4).

Raised intestinal ALP activities were demonstrated in three patient groups: diabetes mellitus, chronic renal failure and intrahepatic obstruction. The highest activities were found in the last group, with activities greater than 120 U/L in two patients. The diagnoses in these two patients were alcoholic cirrhosis and alcoholic hepatitis. In liver disease, the most likely reason for the elevated levels is reduced clearance of intestinal ALP by damaged hepatocytes (Stolbach et al, 1967). Only a small number of patients with diabetes or with chronic renal failure showed biochemical evidence of liver disease. Other factors must be responsible for the raised levels in these patients; in diabetes, for example, it may well be that increased synthesis and release of intestinal ALP occur in response to food intake. However, even in fasting samples, intestinal ALP activities in diabetics were higher than those in healthy adults. Glycation of plasma proteins, such as albumin and fibrinogen, occurs in diabetics. It is possible that intestinal ALP also exists in a glycated form and that this form is cleared more slowly from plasma. It is important to mention that glycation occurs at a slow rate; this, together with the short half-life of plasma intestinal ALP, means that glycation, on its own, is unlikely to be the cause of the raised enzyme activities. Metabolites, retained in the plasma of patients with chronic renal failure, may somehow interact with intestinal ALP, giving rise to an "abnormal" form which is cleared more slowly by the hepatocytes. Moreover, this "abnormal" intestinal ALP may not bind as strongly as the normal form to group A red cells, thus explaining the high activities found in some group A patients.

The diseases (diabetes mellitus, chronic renal failure and liver disease), where raised intestinal ALP activities were present, are all associated with disturbed fat metabolism.

These patients can have raised plasma levels of cholesterol, triglycerides and free fatty acids, with abnormalities in lipoprotein fractions. Intestinal ALP and chylomicrons share the same pathway of entry into the general circulation and are both cleared by the liver. The raised intestinal ALP activities in these patients may be the result of a general abnormality in the transport and clearance of substances carried by the lymphatic system. It would be interesting to see if patients with primary hyperlipidaemias, especially those associated with raised chylomicrons, show abnormalities in plasma intestinal ALP activities.

The finding of a raised plasma total ALP activity in patients is a non-specific indicator of disease. Quantitative measurements of each of the ALP forms in plasma will define a liver and/or bone source of the raised total ALP. This, in turn, should lead to a more meaningful series of tests (radiology or histology) aimed at elucidating the primary cause of the disease. In the small number of patients where intestinal ALP alone is the cause of the raised total ALP and where intrahepatic disease has been excluded, no further investigation is needed.

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PUBLICATIONS

Poster

A modified electrophoretic method for measuring bone alkaline phosphatase

L TIBI and A F SMITH
From the Department of Clinical Chemistry, The Royal Infirmary, Edinburgh EH3 9YW, UK

Introduction

The use of neuraminidase to enhance the electrophoretic separation of liver and bone alkaline phosphatase (ALP) by slowing the mobility of the bone isoenzyme has been reported.[1] However, the retarded bone isoenzyme often overlaps any instestinal isoenzyme also present in the serum, thereby interfering with the assay for bone ALP. Using polyacrylamide-gel electrophoresis in a simple adaptation of the method, we have used a monoclonal antibody to intestinal ALP to overcome this interference.

Methods

TOTAL AND INTESTINAL ALP

Total ALP was measured using *p*-nitrophenyl phosphate as subtrate[2] on a Cobas Bio (Roche, Welwyn Garden City, Hertfordshire). Intestinal ALP was measured using the same conditions as total ALP but in the presence of L-*p*-bromotetramisole[3] and correcting results for the partial inhibition of intestinal ALP activity and residual liver/bone ALP activity. The between-batch coefficients of variation (CV) for total ALP and intestinal ALP were 3.4% and 6.1% respectively.

BONE ALP

Monoclonal antibody to intestinal ALP was kindly provided by Sir Walter Bodmer, Imperial Cancer Research Fund, Lincoln's Inn Fields, London. Antibody was added to plasma samples to give a final dilution of 1 in 200. Samples were incubated with neuraminidase for 15 min at 37°C[1] and then subjected to polyacrylamide-gel electrophoresis.[4] The liver and bone bands were scanned on an Appraise scanner (Beckman, High Wycombe, Bucks) and results for bone ALP expressed as U/L by multiplying the percentage of bone ALP by (total − intestinal ALP). The bone isoenzyme was also measured by heat-inactivation.[5]

All measurements were made in duplicate.

Correspondence: L Tibi, Department of Clinical Chemistry, The Royal Infirmary, Edinburgh EH3 9YW.

Results

Total ALP activity of antibody diluted 1 in 200 was 4 U/L. On electrophoresis, the antibody migrated to the intestinal band position.

The figure shows how intestinal ALP will interfere in bone ALP measurement if neuraminidase treatment is used alone (*b*). The use of the antibody retards intestinal ALP allowing the bone band to be scanned separately (*d*).

Plasma samples ($n = 26$) were run on different gels to assess between-batch precision of bone ALP measurement. These samples had a mean total ALP of 121 U/L (range 66–315 U/L) and a mean intestinal ALP of 28 U/L (range 6–93 U/L). The CV for bone ALP measurement was 4.2% (mean bone ALP = 48 U/L, range = 14–161 U/L). A single plasma sample (mean total ALP = 173 U/L, mean intestinal ALP = 31 U/L) was run on 10 different gels. The CV for bone ALP was 3.3% (mean bone ALP = 108 U/L, SD = 3.6).

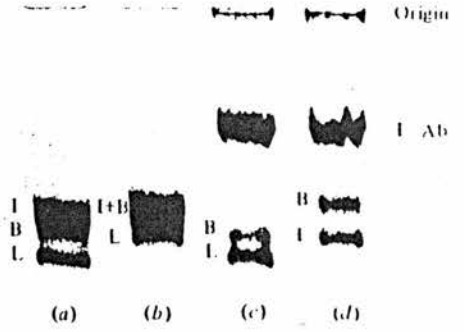

FIG. Separation of ALP isoenzymes without antibody (*a*, *b*) and with antibody (*c*, *d*). Samples (*b*) and (*d*) were pre-treated with neuraminidase. L, Liver ALP; B, bone ALP; I, intestinal ALP; I–Ab, intestinal ALP antibody complex.

Bone ALP in 18 plasma samples (mean total ALP = 209 U/L, mean bone ALP by electrophoresis = 109 U/L) was measured by the modified electrophoretic method and by heat-inactivation. The between-batch CV for heat-inactivation was 8.8% ($n = 15$). Regression analysis gave a correlation coefficient (r) of 0.984 (x = heat-inactivation, y = modified electrophoretic method, intercept = −8.1 U/L, slope = 1.0).

Discussion

Intestinal ALP may be present in plasma under physiological or pathological conditions.[6] In these cases, interference in bone ALP measurement may occur when neuraminidase is used to enhance the separation between bone and liver ALP. The use of antibody prior to neuraminidase treatment is simple and ensures that only bone ALP is measured. The method correlates well with results obtained by the heat-inactivation method for determining bone ALP activity although, in our hands, the heat-inactivation method is less precise.

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Plasma alkaline phosphatase isoenzymes in diabetes mellitus

L. Tibi [a], A. Collier [b], A.W. Patrick [b], B.F. Clarke [b] and A.F. Smith [a]

[a] *University Department of Clinical Chemistry and* [b] *Diabetic and Dietetic Department, The Royal Infirmary, Edinburgh, Scotland (UK)*

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Key words: Alkaline phosphatase isoenzyme; Diabetes; Blood group; Secretor status

Summary

Alkaline phosphatase (ALP) isoenzymes were measured in type 1 diabetics, type 2 diabetics and in a non-diabetic control group. Within the diabetics and the control group, intestinal ALP activity was significantly higher in BO secretors than A secretors or ABO non-secretors. There was no difference in intestinal ALP activity between type 1 and type 2 diabetics but the diabetics had a significantly higher activity of this isoenzyme than the corresponding blood group/secretor status category of the control group. Liver ALP was significantly higher in the diabetics compared with the control group. Bone ALP showed no significant difference between the diabetics and the control group.

Introduction

An increase in total alkaline phosphatase (ALP E.C. 3.1.3.1) activity has been reported in plasma from patients with diabetes mellitus [1,2] but there have been conflicting reports regarding the source of this raised ALP. Some workers [3,4] have found a high occurrence of abnormal liver function tests in diabetics suggesting a liver source for the raised total ALP. Stepan et al [5] measured liver and bone isoenzymes in a series of diabetic patients. They found that the increase in total activity of serum ALP was due to the bone isoenzyme in 39% of their patients. In 19% of these cases there was a simultaneous increase in the liver isoenzyme. Maxwell et al [6] also found an abnormally high bone isoenzyme fraction in the plasma of diabetics.

Correspondence to: L. Tibi, University Department of Clinical Chemistry, The Royal Infirmary, Edinburgh EH3 9YN, Scotland, UK.

We have measured liver, bone and intestinal ALP in the plasma of type 1 and type 2 diabetic patients and compared the levels with those found in the plasma of healthy volunteers. Blood group and secretor status of the diabetics and the volunteers were also determined because of the association of these with intestinal ALP [7].

Patients

Patients and controls

Patients were recruited from those routinely attending the Diabetic Department, Royal Infirmary of Edinburgh; the first 5 to 12 out-patients at each morning clinic were asked to participate. Patients who had type 1 disease were classified by insulin dependence, their clinical history and family history of the disease. The patients who had type 2 disease were not dependent on insulin. The control group was made up of 44 healthy volunteers, all of whom where hospital workers. Details of the diabetic and control groups are given in Table I.

A non-fasting venous blood sample and a saliva sample were collected from each patient and control. The time of sampling for the patients and controls was mid-morning. The separated plasma and the saliva were kept at $-20\,^{\circ}C$ until analysis.

Methods

Total and intestinal ALP

Total ALP was measured using 16 mmol/l *p*-nitrophenyl phosphate in 2-amino-2-methyl-1-propanol buffer, pH 10.5 [8]. The absorbance change was monitored at 405 nm at 37 °C on a Cobas Bio analyser (Roche, Welwyn Garden City, UK). Intestinal ALP was measured using the same conditions as for total ALP but in the presence of 50 μmol/l L-*p*-bromotetramisole [9]. The results were corrected for partial inhibition of intestinal ALP activity (13.54%) and residual liver/bone ALP activity (13.93%) in the presence of L-*p*-bromotetramisole. The between-batch coefficient of variation (CV) for total ALP was 3.4% (mean total ALP = 73 U/l, range = 23–132 U/l, $n = 13$). The between-batch CV for intestinal ALP was 6.1% (mean intestinal ALP = 22 U/l, range = 3–44 U/l, $n = 13$).

Electrophoresis

Bone and liver ALP were separated by polyacrylamide-gel electrophoresis [10]. Prior to electrophoresis, samples were incubated with neuraminidase (15 min at 37 °C) to enhance the separation of liver and bone ALP [11]. Intestinal ALP interferes in the measurement of bone ALP if neuraminidase treatment is used alone since the retarded bone ALP has a mobility similar to that of intestinal ALP. We therefore added to samples a monoclonal antibody to intestinal ALP (1 : 200) prior to neuraminidase incubation. This complexes and retards intestinal ALP allowing the bone band to be quantitated separately. Gels were scanned on an Appraise scanner (Beckman, High Wycombe, Bucks) and results for bone and liver ALP

TABLE I

Details of the diabetic and control groups

	Number			Age (yr)			Duration of diabetes (yr)		
	Total	Male	Female	Mean	SD	Range	Mean	SD	Range
Type 1	83	48	35	39.5	13.3	20–65	7.1	5.8	1–22
Type 2	83	49	34	56.0	6.8	38–65	11.7	8.2	1–45
Control group	44	18	26	35.6	11.1	20–63	–	–	–

expressed in U/l by multiplying the percentage values obtained for each peak by (total – intestinal ALP). The between-batch CV for bone ALP measurement was 4.2% (mean bone ALP = 48 U/l, range = 15–161 U/l, $n = 26$). For liver ALP, the between-batch CV was 4.6% (mean liver ALP = 45 U/l, range = 12–236 U/l, $n = 26$).

The biliary band remains at the origin on polyacrylamide-gel electrophoresis. There was minimal staining at the origin in all the samples studied; when biliary ALP was quantitated by gel filtration it accounted for < 3% of total ALP.

Total, intestinal, liver and bone ALP measurements were made in duplicate.

Liver function tests

Bilirubin, alanine aminotransferase (ALT, EC 2.6.2.2) and gamma-glutamyltransferase (GGT, EC 2.3.2.2) were measured on a continuous flow analyser (SMAC II; Technicon Instrument Corp., Basingstoke, UK). The reference interval for bilirubin is 2–17 μmol/l, for ALT it is 10–40 U/l and for GGT it is 10–55 U/l (males) and 10–35 U/l (females).

Glycated haemoglobin

Glycated haemoglobin (HbA_1) was measured using commercially available agar plates (Corning Medical, Halstead, UK). The reference interval for HbA_1 is 4.5–8%.

Blood groups and secretor status

The ABO and Lewis blood groups were determined by agglutination and the secretor status by haemagglutination inhibition using saliva [12]. The Lewis blood group was used to confirm the results obtained from the saliva sample.

The results of these studies have been used to place the diabetics and controls into three categories: (1) Group O secretors and group B secretors – 'BO secretors'. (2) Group A secretors – 'A secretors'. (3) Group A, group B and group O non-secretors – 'ABO non-secretors'. The group A secretors have been separated from the other secretors because previous reports have indicated that in terms of intestinal ALP activity following a fatty meal they behave as 'non-secretors'.

Statistical analysis

A non-parametric test (Mann–Whitney U test) was used to compare the results in the diabetics with those in the control group and to compare different categories according to secretor status.

Results

Mean HbA_1 of type 1 diabetics was 10.9% (SD 2.1, range 6.6–17.5%) and of type 2 diabetics 10.3% (SD 2.7, range 6.1–20.7%).

The numbers of diabetics and controls with bilirubin, ALT, GGT and total ALP above the reference intervals for our laboratory are given in Table II.

Table III shows the mean, SD and range of total and intestinal ALP in the diabetics and controls. Figure 1 shows the data for intestinal ALP. Within all groups

TABLE II

Number (percentage) of diabetics and of controls with results for bilirubin, ALT, GGT and total ALP above the reference range

	n	Bilirubin	ALT	GGT	Total ALP
Type 1	83	8 (9.5)	3 (3.5)	4 (5.0)	7 (8.5)
Type 2	83	6 (7.0)	18 (21.5)	26 (31.5)	10 (12.0)
Control group	44	2 (4.5)	2 (4.5)	1 (2.5)	0 (0)

of patients and within the control group there was no significant difference in either total ALP or intestinal ALP between A secretors and ABO non-secretors. On the other hand, within each diabetic group and within the control group both total ALP and intestinal ALP activities were higher in the BO secretors than in A secretors and ABO non-secretors ($p < 0.05$ in all cases).

The activities of intestinal ALP of BO secretors of type 1 and type 2 diabetics did not differ significantly from one another but both were significantly higher than BO secretors of the control group ($p < 0.0001$). Similarly, A secretors and ABO non-secretors of the diabetics had significantly higher intestinal ALP activity than A secretors and ABO nonsecretors of the control group ($p < 0.0001$).

TABLE III

Total and intestinal ALP activity of type 1, type 2 diabetics and of the control group [a]

	Total ALP (U/l)			Intestinal ALP (U/l)		
	BO secretors	A secretors	Non-secretors	BO secretors	A secretors	Non-secretors
Type 1						
n	35	15	33	35	15	33
$\bar{x}$	79.5	67.7 *	59.7	27.2 **	9.3 **	8.2 **
SD	24.4	20.8	15.5	13.5	3.5	3.9
Range	48–162	32–109	39–92	9–64	3–19	3–19
Type 2						
n	36	23	24	36	23	24
$\bar{x}$	85.9 *	65.0 **	70.7 *	30.0 **	8.6 **	9.3 **
SD	32.1	11.6	21.3	22.2	2.7	3.7
Range	40–137	47–86	42–128	7–106	5–15	5–20
Control group						
n	18	11	15	18	11	15
$\bar{x}$	67.4	48.1	51.9	13.0	4.6	4.1
SD	12.0	7.8	15.7	4.9	1.6	1.4
Range	36–91	35–62	35–69	5–25	2–8	2–6

[a] Statistical tests were done comparing the diabetics (type 1 or 2) with the control group.
[b] Asterisks indicate the level of significance (* $p < 0.01$, ** $p < 0.001$).

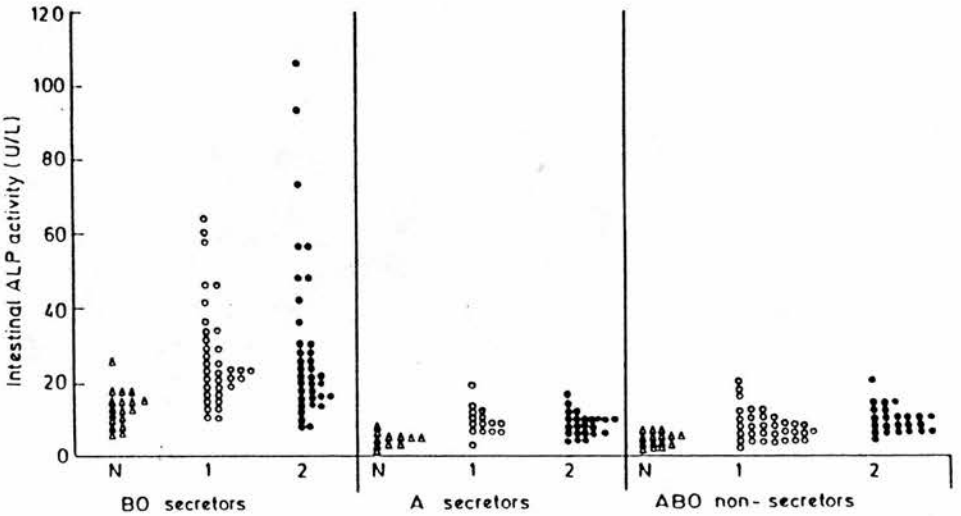

Fig. 1. Plasma intestinal ALP activity (U/l) in the control group (N-△), type 1 diabetics (1-○), type 2 diabetics (2-●) for the three blood groups/secretor status categories (BO secretors, A secretors and ABO non-secretors).

Eight diabetics (all O secretors) with intestinal ALP activity > 40 U/l were selected. Fasting samples were taken from these patients at the next clinic visit. In all cases, intestinal ALP was significantly lower ($p < 0.01$) in the fasting compared

TABLE IV

Liver and bone ALP activity of type 1, type 2 diabetics and of the control group [a]

	Liver ALP (U/l)	Bone ALP (U/l)
Type 1		
n	83	83
$\bar{x}$	25.9 *	27.1
SD	13.5	8.6
Range	8–78	15–52
Type 2		
n	83	83
$\bar{x}$	29.0 **	28.7
SD	10.7	11.1
Range	11–59	14–63
Control group		
n	44	44
$\bar{x}$	20.6	29.0
SD	7.3	7.0
Range	8–45	17–45

[a] Statistical tests were done comparing the diabetics (type 1 or 2) with the control group.
[b] Asterisks indicate the level of significance (* $p < 0.05$, ** $p < 0.001$).

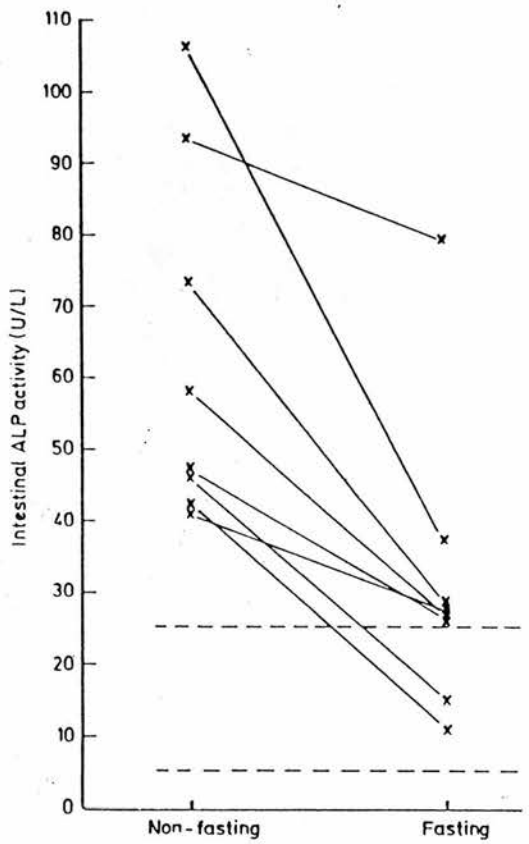

Fig. 2. Intestinal ALP activity (U/l) of non-fasting and fasting plasma samples from 8 diabetics. The dotted lines show the range of intestinal ALP activity found for BO secretors of the control group.

to the non-fasting sample (mean fasting intestinal ALP = 31.1 U/l, SD = 20.9, range = 11–79 U/l; mean non-fasting intestinal ALP = 63.3 U/l, SD = 24.9, range = 41–106 U/l). Intestinal ALP activity of the non-fasting and fasting samples of the eight diabetics are given in Fig. 2. The dotted lines show the range of intestinal ALP activity found in the BO secretors of the control group. In most cases, the fasting sample from the diabetics had a higher intestinal ALP activity than was found in the samples from the control group.

Table IV shows the mean, SD and range of liver and bone ALP of the diabetics and the control group. Both type 1 and type 2 diabetics had significantly higher liver ALP than the control group ($p < 0.05$). Of the eight diabetics with liver ALP > 50 U/l, two had GGT activities above the reference range. Type 2 diabetics had higher liver ALP than type 1 diabetics ($p < 0.01$). For bone ALP there was no statistical difference between the groups.

Discussion

Intestinal ALP appears in the plasma of all subjects after ingestion of fat but not after carbohydrate or protein [13]. The magnitude of the rise in intestinal ALP is dependent on secretor status—secretors showing a greater increase in intestinal ALP activity after a fatty meal than non-secretors [14]. Blood group A secretors behave differently from group B and O secretors in that their increases in intestinal ALP activity after a fatty meal are less marked. Lower activities of intestinal ALP in persons of blood group A has been explained by the greater binding of intestinal ALP to red cells of blood group A compared with red cells of blood groups B or O [15].

Although both total and intestinal ALP were higher, within both diabetic and control groups, in BO secretors than in A secretors or non-secretors, there were also differences between the diabetics and the control group. Most notably intestinal ALP activity was higher within each blood group/secretor category in diabetics than in the non-diabetic control group. The magnitude of the increased activity of intestinal ALP was sufficient to explain the raised plasma total ALP activity in 11 out of 17 patients with raised plasma total ALP activity. These patients were all BO secretors. The use of fasting samples resulted in a reduction in intestinal ALP but in most cases not to levels found in the control group. Thus the high levels of intestinal ALP may be partly explained by an abnormal response in the diabetics to food intake.

Six of the 14 patients with raised intestinal ALP (> 40 U/l) had an associated abnormality of GGT or ALT. Intestinal ALP has been reported to be raised in patients with cirrhosis [16] and this is thought to be due to impaired clearance of intestinal ALP by the liver. Disturbed liver function may explain the raised intestinal ALP in some of the diabetics.

Liver ALP in type 1 and type 2 diabetics was higher than the controls. However, type 2 diabetics had a higher mean liver ALP compared with type 1 diabetics (Table IV). There was also a greater number of abnormal results for ALT and GGT in type 2 diabetics compared with type 1 diabetics (Table II). Both these findings suggest a greater incidence of liver disease in type 2 diabetics than in type 1.

Bone ALP was not significantly different in the three groups. Bone ALP accounted for most of the increase in total ALP above the reference range in three diabetics. Our findings differ from Stepan et al [5] who found that bone ALP accounted for the increase in total ALP above the reference range in 39% of their series of diabetic patients.

In conclusion, we find that when a non-fasting blood sample is used intestinal ALP is an important source of raised total ALP in diabetics who are BO secretors. Intestinal ALP is reduced in fasting samples but not to the level of BO secretors of the control group.

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Alkaline Phosphatase Isoenzymes in Plasma in Hyperthyroidism

L. Tibl, A. W. Patrick, P. Leslie, A. D. Toft, and A. F. Smith

Alkaline phosphatase (ALP; EC 3.1.3.1) isoenzymes were measured in the plasma of 63 untreated hyperthyroid patients (the hyperthyroid group), 58 treated hyperthyroid patients, and 100 blood donors. Total, liver, and bone ALP activities were significantly higher in the hyperthyroid group than in the treated hyperthyroid group or the blood donors. Bone ALP was more frequently and more markedly abnormal than liver ALP. Intestinal ALP did not differ significantly between the groups. The hyperthyroid patient group had significantly higher plasma calcium concentrations and lower serum parathyrin concentrations than those of the treated hyperthyroid group.

Additional Keyphrases: *calcium · parathyrin · markers of liver disease*

Total alkaline phosphatase (ALP; EC 3.1.3.1) activities exceeding the normal reference range have been reported in serum from patients with hyperthyroidism (*1, 2*).[1] Quantification of ALP isoenzymes has shown this increase to be of only liver origin in some patients, of only bone origin in other patients, and sometimes to be of both liver and bone origin (*2*). We have measured liver, bone, intestinal, and total ALP in plasma samples from a group of untreated hyperthyroid patients and compared the activities with those in plasma from treated thyrotoxic patients and blood donors. Blood group and secretor status of the patients and the blood donors were determined because of the association of these with intestinal ALP in serum (*3*). We correlated our findings for liver and bone ALP with other biochemical indices of liver and bone disease.

Materials and Methods

Patients and Controls

Group 1. Hyperthyroid patients. Patients in this group were attending the Thyroid Clinic for investigation of thyroid disease. Patients who were clinically and biochemically hyperthyroid (plasma TSH <0.1 milli-int. unit/L, free T_4 >23 pmol/L) and who had not begun treatment were selected. Details about the patients are given in Table 1.

Blood samples (lithium heparin-treated and clotted blood) and a saliva sample were collected from each patient. Serum (for PTH assay) was separated and frozen within an hour. The plasma and saliva samples were kept at −20 °C until analysis.

Group 2. Euthyroid patients. The patients in this group had been previously thyrotoxic but at the time of sampling were clinically and biochemically euthyroid (plasma TSH and free T_4 within the reference range). One patient had exophthalmic Graves' disease.

University Departments of Clinical Chemistry and Medicine, The Royal Infirmary, Edinburgh EH3 9YW, Scotland, U.K.

[1] Nonstandard abbreviations: ALP, alkaline phosphatase; TSH, thyrotropin; T_4, thyroxin; PTH, parathyrin; ALT, alanine aminotransferase; GST, glutathione *S*-transferase; and GGT, γ-glutamyltransferase.

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Table 1. Age and Sex Distribution of Patients in Group 1 and Group 2

	No.			Age, years		
Group	Total	Male	Female	Mean	SD	Range
1	63	10	53	52	13.4	22–85
2	58	10	48	47	15.1	19–81

Treatment consisted of radioactive iodine (n = 35), thyroidectomy (n = 15), or carbimazole (n = 7). The mean time since treatment was 6.5 years (SD = 5.9 years, range 0.5–32 years). Twenty-seven patients were taking T_4; the remainder were on no replacement therapy. Samples were collected as for group 1. Patients are described in Table 1.

Group 3. Control group. Samples from 100 blood donors were supplied by the Regional Blood Transfusion Service, Scotland. The age and sex of the donors were not documented. Serum was used for ALP isoenzyme analysis and the remaining blood for determination of ABO and Lewis blood groups.

Methods

Total ALP and ALP isoenzymes. Total ALP was measured with 16 mmol/L *p*-nitrophenyl phosphate substrate in 2-amino-2-methyl-1-propanol buffer, pH 10.5 (*4*). The absorbance change was monitored at 405 nm with a Cobas-Bio analyzer (Roche, Welwyn Garden City, U.K.). The between-batch coefficient of variation (CV) was 1.6% (mean total ALP = 112 U/L, range = 62–214 U/L, n = 24). Intestinal ALP was measured by a method involving coating microtiter plates with monoclonal antibody to intestinal ALP and measuring ALP activity after addition of plasma (*5*). The between-batch CV for intestinal ALP measurement was 6.1% (mean intestinal ALP = 11.3 U/L, range = 2.8–28.9 U/L, n = 18). Liver and bone ALP were separated by polyacrylamide-gel electrophoresis and quantified by scanning as described previously (*6*). The between-batch CV for liver ALP measurement was 4.7% (mean liver ALP = 43 U/L, range = 16–77 U/L, n = 24). The between-batch CV for bone ALP measurement was 3.9% (mean bone ALP = 52 U/L, range = 18–129 U/L, n = 24). Measurements for total, liver, and bone ALP were made in duplicate; intestinal ALP measurements were made in triplicate. The biliary form of ALP, which appears in patients with cholestasis and remains at the origin on polyacrylamide-gel electrophoresis, was not quantified, there being only slight staining at the origin in all the samples.

Blood groups and secretor status. The ABO and Lewis blood groups were determined by agglutination, and the secretor status by hemagglutination inhibition, with saliva samples (*7*). The Lewis blood group was used to confirm the results obtained from the saliva samples from the patients (groups 1 and 2).

Other measurements. Plasma TSH and free T_4 were assayed with a commercially available kit (SimulTRAC; Becton Dickinson, Orangeburg, NY).

Serum PTH was measured in 46 of the group 1 patients

and in 39 of the group 2 patients, with use of the Allegro Intact PTH kit (from Nichols Institute; obtained via Biogenesis Ltd., Bournemouth, U.K.).

Plasma glutathione *S*-transferase (hepatic GST B_1B_1) was measured by radioimmunoassay (*8*).

Concentrations of alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), albumin, and calcium in plasma were measured with a continuous-flow analyzer (SMAC II; Technicon Instruments Corp., Basingstoke, U.K.).

Statistical tests. Standard parametric tests were used to compare group means (*t*-test or analysis of variance, as appropriate) and to measure correlations between variables. A Mann–Whitney test was used where the data were not approximately normally distributed. The data were analyzed with use of the statistical package SPSS/PC+ on an IBM PS/2 Model 70.

Results

Thyroid-function tests. Patients in group 1 had TSH concentrations <0.1 milli-int. unit/L and a mean free T_4 of 56 pmol/L (SD = 24.9, range = 24–123 pmol/L). Group 2 patients had a mean TSH of 1.9 milli-int. units/L (SD = 1.3, range = 0.3–4.9 milli-int. units/L) and a mean free T_4 of 15 pmol/L (SD = 3.9, range = 9–24 pmol/L).

Total ALP and isoenzymes. Concentration of total ALP, liver ALP, and bone ALP were all higher in group 1 than in group 2 or in the control group (P <0.001 in all cases) (Table 2). Group 2 also showed higher activities for total ALP (P <0.005), bone ALP (P <0.02), and liver ALP (P <0.01) than did the control group. Individual values for the three groups are shown in Figure 1.

Because of the known association between intestinal ALP and blood group and secretor status, we divided patients and controls into three categories:

Group B, O secretors	(Lewis b)
Group A secretors	(Lewis b)
Group AB and A, B, O nonsecretors	(Lewis a)

Group A secretors were separated from other secretors because, in terms of increases of intestinal ALP activity in plasma after a fatty meal, these subjects behave the same as nonsecretors. There was no difference in intestinal ALP activity in group 1, group 2, or the control group, either when all categories were considered together or when Group B, O secretors were considered alone (Table 3). Group A secretors and nonsecretors of group 1 and 2 showed higher concentrations of intestinal ALP than did the control group.

Within the treated thyrotoxic group, we found no correlation between total, liver, bone, or intestinal ALP and the time since treatment of thyrotoxicosis. Patients treated with carbimazole had significantly lower (P <0.005) total and liver ALP concentrations than did patients treated with radioactive iodine. (This may be because of patient

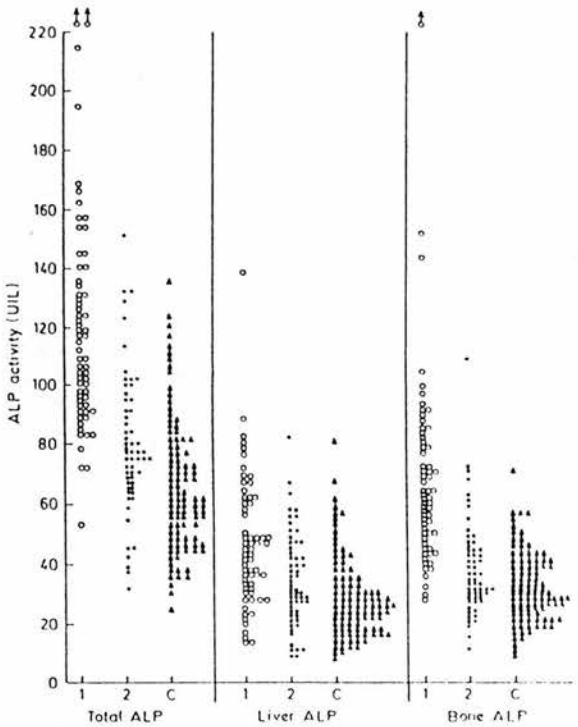

Fig. 1. Total, liver, and bone ALP activities in group 1 (○), group 2 (●), and the control group (▲)

Table 2. Total, Liver, and Bone ALP of Group 1, Group 2, and the Controls

	ALP, U/L		
	Total	Liver	Bone
Group 1			
$\bar{x}$	124	46.9	69.3
SD	51.5	22.0	42.1
Range	53–407	14–138	28–340
Group 2			
$\bar{x}$	80	35.4	38.7
SD	24.1	15.7	16.4
Range	32–151	9–82	10–109
Controls			
$\bar{x}$	67.4	28.8	32.7
SD	21.8	13.3	11.7
Range	26–135	10–80	10–71

Table 3. Intestinal ALP of Group 1, Group 2, and the Controls

	Intestinal ALP, U/L			
	All	B, O secretors[a]	A secretors	Nonsecretors
Group 1				
n	63	28	15	20
$\bar{x}$	7.3	11.5	4.0	3.7
SD	5.0	4.6	1.5	0.9
Range	3–22	5–22	3–8	3–7
Group 2				
n	58	19	16	21[a]
$\bar{x}$	6.3	10.0	4.4	3.8
SD	4.3	5.0	1.4	0.7
Range	2–23	3–23	2–7	3–6
Controls[b]				
n	100	44	23	28[b]
$\bar{x}$	5.9	9.1	3.0	2.9
SD	4.8	5.3	0.7	0.6
Range	2–23	3–23	2–4	2–4

[a] AB secretors not included.
[b] Lewis a⁻b⁻ subjects not included.

selection for each treatment category.) There was no correlation between ALP variables and T_4 treatment dose.

Liver-related measurements. Table 4 gives the mean concentrations of bilirubin, ALT, GGT, and GST measured in group 1 and 2 patients, and the reference intervals for these analytes in this laboratory. In all cases, results were significantly higher ($P < 0.05$) in group 1 than in group 2.

Bone-related measurements. Group 1 patients had higher mean concentrations of calcium and a lower albumin concentration in plasma than did group 2 (see Table 4). The differences were all statistically significant ($P < 0.05$). The difference between the two groups in ionized calcium in plasma is probably greater than the difference in total calcium because albumin concentrations in group 1 were lower than those in group 2.

Figure 2 shows the concentrations of calcium in plasma and of PTH in serum from group 1 and 2 patients. The higher mean calcium in group 1 was associated with a significantly lower mean PTH in group 1 vs group 2. The plasma creatinine concentration of patients with PTH concentrations >55 ng/L was within the normal reference interval.

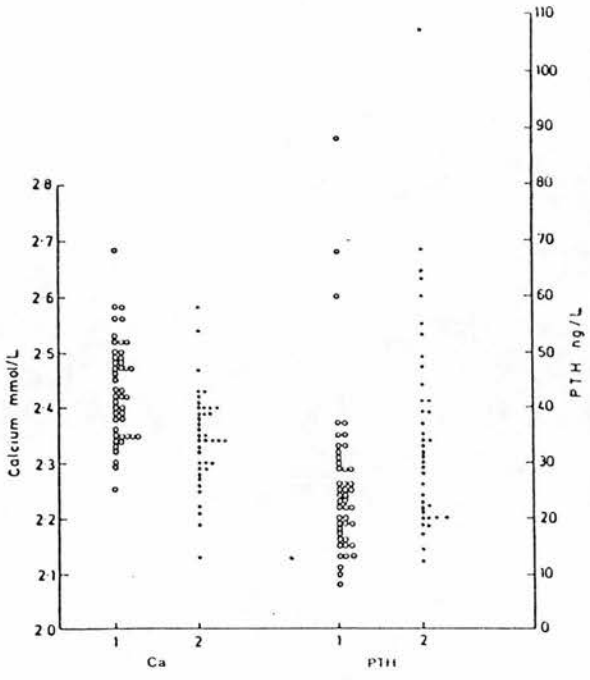

Fig. 2. Plasma calcium and serum PTH in group 1 (○) and group 2 (●) patients

Discussion

Total ALP activity was increased in 51% of untreated hyperthyroid patients (we used total ALP of 110 U/L as the cutoff, because 95% of the control group had values below this). Our results compare well with those of Cooper et al. (*1*), who found ALP increases in almost half of their series of thyrotoxic patients. However, Rhone et al. (*2*) found 77% of thyrotoxic patients had ALP >110 U/L. We found no correlation between ALP and free T_4, unlike Cooper et al. (*1*), who found a significant correlation between ALP and T_4.

We have found that 30% of untreated hyperthyroid patients (group 1) had increased liver ALP activities (cutoff, 55 U/L) and that the mean liver ALP activity concentration of group 1 was significantly higher than that of the treated thyrotoxic group (group 2) (Table 2). Rhone et al. (2) found that 63% of those in their study of untreated thyrotoxic patients had above-normal concentrations of liver ALP. The difference between the findings of the two studies may be explained by a difference in the severity of thyrotoxicosis or by a difference in the methods of isoenzyme measurement. Other indices of liver disease (bilirubin, ALT, GGT, and GST) were also higher in group 1 (Table 4). Other workers have found ALT activities within the reference range (2), increased GGT (*9*), and increased GST (*10*) in hyperthyroidism. We found a positive correlation between free T_4 and GST in group 1 patients, but no correlation between free T_4 and bilirubin, ALT, GGT, or liver ALP.

Our results indicate that patients with untreated hyperthyroidism show evidence of liver dysfunction, although in most cases this is minor. Comparison with group 2 patients suggests that the abnormalities largely resolve on treatment of hyperthyroidism. These previously hyperthyroid patients, although now euthyroid, had significantly higher concentrations of liver ALP than did the control group (Table 2); the range and distribution of activities in the two groups was similar (Figure 1).

In our study, 63% of hyperthyroid patients had increased bone ALP (>55 U/L). This compares well with the results of Rhone et al. (2), who found that 68% of their patients had activities exceeding those in control sera. The distribution and range of bone ALP in group 2 were similar to those in the control group (Figure 1), apart from one patient who

Table 4. Concentrations of Bilirubin, ALT, GGT, GST, Albumin, Calcium, and PTH in Groups 1 and 2

	Bilirubin, μmol/L	ALT, U/L	GGT, U/L	GST, μg/L	Albumin, g/L	Calcium, mmol/L	PTH, ng/L
Reference interval	2–17	10–40	10–55 (♂) 5–35 (♀)	<4.5	36–47	2.12–2.62	10–55
Group 1							
$\bar{x}$	9.8	32	36	5.4	43	2.44	25.9
SD	3.5	16.9	35.5	3.1	2.8	0.09	14.7
Range	6–22	10–102	6–217	1–19	37–48	2.29–2.68	8–88
Group 2							
$\bar{x}$	6.9	18	16	2.7	45	2.33	35.6
SD	2.7	6.5	10.6	1.3	2.1	0.09	18.8
Range	3–12	10–37	5–63	1–6	40–49	2.09–2.58	12–107

had been treated with radioactive iodine six months previously, whose bone ALP activity was 108 U/L. The high bone ALP activity in this case is consistent with the findings of Cooper et al. (*1*), who showed an increase in ALP activity (predominantly of bone origin) after treatment with radioactive iodine, with a peak at three months afterwards.

The increased bone ALP activity in hyperthyroidism can be explained by a direct action of thyroid hormones on osteoblasts (*11*). We could not demonstrate a correlation between free T_4 and bone ALP activity. In addition to increased osteoblastic activity, there is increased osteoclastic bone resorption, with increased plasma and urinary concentrations of calcium (*12*). We found higher mean concentrations of calcium in plasma from group 1 than from group 2, although nearly all values were within the reference range (Figure 2).

The physiological response to increases in circulating calcium concentrations is suppression of PTH release from the parathyroid glands. The assay we used for PTH measurement is sensitive and measures the intact, biologically active molecule. Mean PTH was lower in group 1 than in group 2 (Figure 2), which supports the hypothesis that some agent other than PTH is responsible for the increase of calcium in plasma in hyperthyroidism. Three patients in group 1 and five patients in group 2 had PTH concentrations above the normal reference range. These patients were normocalcemic, with normal renal function; we could find no explanation for their results.

Intestinal ALP activity was not different between group 1, group 2, and the controls when Group B, O secretors were considered alone (Table 3). Nonspecific interference by nonintestinal ALP in the coated-plate method may explain the slightly higher intestinal ALP values in Group A secretors and nonsecretors of groups 1 and 2, as compared with the control group.

To conclude: Total ALP activity was increased in approximately half of our untreated hyperthyroid patient group, but the abnormalities in liver and bone ALP were largely resolved on treatment. Thus, in most cases, the finding of increased ALP in an untreated hyperthyroid patient would not seem to require further investigation, unless the finding persists after the patient becomes euthyroid.

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